Unraveling the structure of soil food webs: stable isotope, fatty acid and compound-specific fatty acid analysis

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Contents

Zusammenfassung

In dieser Arbeit wurden bereits vorhandene Methoden zur Untersuchung von Bodennahrungsnetzen untersucht und neue Methoden bewertet. Biota im Boden bestehen aus mehr Arten als in jedem anderen Habitat der Erde. Trotz seiner enormen Bedeutung ist es eines der am schlechtesten verstandenen Systeme. Um Bodennahrungsnetze zu untersuchen benötigt man indirekte Methoden, da Bodenorganismen aufgrund ihrer geringen Größe und ihrer kryptischen Lebensweise schwer direkt zu beobachten sind.

Eine weit verbreitete Methode zur Untersuchung von Nahrungsnetzen ist die Analyse der Variation von natürlich vorkommenden stabilen Isotopen, vor allem von $^{15}N/^{14}N$ und $^{13}C/^{12}C$. Stickstoffisotope werden dabei zur Bestimmung der trophischen Ebene genutzt und Kohlenstoffisotope zur Bestimmung der Nahrungsquelle. Im Allgemeinen geht man von einer Anreicherung des schweren Stickstoffisotops von 3,4‰ (δ-Einheiten) pro trophischer Ebene aus und von 1‰ für das schwerere Kohlenstoffisotop. Diese Studie zeigte jedoch eine Variation zwischen 2,4 und 6,3‰ für ^{15}N und zwischen -1,0 und -3.3‰ für ^{13}C aufgrund von Qualität der Nahrung, Hunger oder dem Alter von Bodentieren (Collembolen). Das zeigt, dass der physiologische Zustand und die Nahrungsqualität einen Einfluss auf die Fraktionierung von stabilen Isotopen hat, der bei der Analyse von Nahrungsnetzen berücksichtigt werden sollte.

Als Alternative zu der Isotopenanalyse wurde die Fettsäureanalyse untersucht. Dies ist eine relativ neue Methode zur Untersuchung von Nahrungsnetzen im Boden. Dabei wird das Fettsäuremuster von Bodentieren analysiert; aus dem Muster und dem Vorkommen bestimmter Biomarker-Fettsäuren kann auf die Nahrungsquelle geschlossen werden. Um diese Methode erfolgreich anwenden zu können, wurde der Einfluss von physiologischen Faktoren und Umweltfaktoren auf Fettsäuremuster von Bodentieren untersucht. Das Fettsäuremuster von Collembolen wurde nicht signifikant von Nahrungsmangel beeinflusst, jedoch von Nahrungsqualität, Lebensstadium und Umgebungstemperatur. Allerdings betrug der spezifische Marker für pilzliche Nahrung (Linolsäure) unter allen getesteten Faktoren über 20% aller Fettsäuren, im Gegensatz zu anderen Nahrungsquellen (2 - 14%). Andere Studien entdeckten Fettsäuremarker für Blätter, Nematoden und Pilze; in der vorliegenden Arbeit wurden spezifische Marker für bakterielle Nahrung identifiziert. Verzweigtkettige Fettsäuren (i14:0, i15:0, a15:0 und i17:0) charakterisieren Collembolen, die mit gram-positiven Bakterien gefüttert wurden, eine cyclische (cy17:0) und 16:1ω5 reflektierte eine Ernährung von gram-negativen Bakterien.

Eine verfeinerte Methode der Fettsäureanalyse ist die komponentenspezifische ^{13}C-

Fettsäureanalyse. Der Vorteil dieser Methode ist, dass das $^{13}C/^{12}C$-Verhältnis in spezifischen Nahrungsmarkern gemessen werden kann. Ein ähnliches $^{13}C/^{12}C$-Verhältnis in derselben Fettsäure in Konsument und potenzieller Nahrung weist auf direkten trophischen Transfer in das Körpergewebe des Konsumenten hin. Die Anwendbarkeit dieser Methode für Nahrungsnetzuntersuchungen im Boden wurde in einem Freilandexperiment getestet. Dabei wurden wesentliche trophische Gruppen untersucht: Mikroorganismen als Primärzersetzer, euedaphische und epedaphische Collembolen als Sekundärzersetzer und vagante und netzbauende Spinnen als Top-Prädatoren. Der Versuch demonstrierte, dass durch $^{13}C/^{12}C$- Verhältnisse einzelner Fettsäuren in potenzieller Beute und in Konsumenten der Kohlenstoffffluss und trophische Verknüpfungen analysiert werden können.

Die vorliegende Arbeit trug zur Etablierung neuer Werkzeuge bei, um das "Mysterium" der Diversität von Tieren im Boden und der Struktur von Bodennahrungsnetzen zu entschlüsseln.

Summary

In this work available methods for soil food web analysis were studied and new methods were evaluated. Soil biota consist of more species than the biota of any other environment on earth, however, despite their importance they are the least understood. Indirect methods are needed to get insight into soil food webs, because of the cryptic habitat and the small size of soil animals.

A common used method is the analysis of the variation of natural occurring stable isotopes, mainly $^{15}N/^{14}N$ and $^{13}C/^{12}C$. Nitrogen isotopes can be used to obtain trophic level information and carbon is used to determine food sources. Commonly the shift per trophic level is considered to be about 3.4‰ for $^{15}N/^{14}N$ and 1‰ for $^{13}C/^{12}C$. But in this study $\delta^{15}N$ varied from 2.4 to 6.3‰ and $\delta^{13}C$ from -1.0 to -3.3‰ due to fungal food quality, starvation or age of soil animals (Collembola). This proves the impact of physiological status and food quality on stable isotope fractionation which should be considered when analysing food webs.

Since the stable isotope method has its drawbacks, fatty acid (FA) analysis as alternative method for analysing soil food webs was investigated. FA analysis is a relatively new approach for investigating soil food webs. The FA pattern or specific biomarker FAs in consumers allow to infer food sources. To successfully apply this method, the influence of physiological and of environmental effects had to be tested. The FA pattern of Collembola was not significantly influenced by starvation, but food quality, life stage and temperature had an impact. Nevertheless, the specific marker FA (linoleic acid) for fungal food source made up over 20% of all FAs at all tested conditions compared to Collembola reared on other diets (2 - 14%). Other studies proved specific FA markers for leaves, nematodes and fungi; the present work revealed specific marker FAs for bacterial diet. Methyl branched FAs (i14:0, i15:0, a15:0 and i17:0) characterized Collembola reared on gram-positive bacteria, and a cyclic form (cy17:0) and 16:1ω5 were characteristic for gram-negative bacteria.

A sophistication of FA analysis is the compound-specific ^{13}C fatty acid analysis. The advantage of this method is that it delivers the $^{13}C/^{12}C$ ratio of specific dietary marker FAs. A similar $^{13}C/^{12}C$ ratio in the same FA in consumer and potential diet indicates trophic transfer and routing of the FA into the tissue of the consumer. The applicability of this analysis for soil food web investigations was tested in a field experiment including major functional groups, i.e. microorganisms as primary decomposers, euedaphic and epedaphic Collembola as secondary decomposers and cursorial and web-building spiders

as top predators. Results of this study demonstrate that $^{13}C/^{12}C$ ratios of individual FAs in potential prey (diet) and consumers allow to identify carbon fluxes and trophic links.

This work contributed to the establishment of tools to unravel the "enigma" of soil animal diversity and of the structure of soil food webs.

Chapter 1

General Introduction

1.1 Structure and function of soil food webs

All terrestrial ecosystems depend on plants, which in turn rely on water and nutrients from the soil (Fitter, 2005). Regardless of this important interaction between above- and belowground components, they have traditionally been considered separately, and the focus was mainly on aboveground processes (Wardle et al., 2004). Only recently the important connection between soil decomposers and aboveground herbivores has been taken into account (Bardgett, 1998, 2005; van der Putten, 2001; Wurst et al., 2004a,b). Aboveground systems are responsible for most of the production (carbon input) in an ecosystem, whereas belowground systems are responsible for most of the decomposition (carbon loss). Soil biota play a major role by recycling 80-90% of the organic matter from plant based food webs, and indirectly regulate plant growth and community composition by determining the supply of available soil nutrients (Wardle et al., 2004). Soil biota consist of more species than the biota of any other environment on earth; despite their importance they are the least understood (Young and Crawford, 2004; Bardgett, 2005).

Fungi and bacteria constitute the major component and the basal trophic level of most food chains in soil, with fungi as most abundant primary decomposer (up to 3 km hyphae per g soil; Bardgett, 2005). Consumers and diet in soil food webs do not coevolve, because they have dead organic matter as basal resource (Scheu and Setälä, 2001). The lack of coevolution has the consequence that detritivores tend to be less specialised than herbivores. Species which are taxonomically unrelated exploit similar resources and most decomposer soil animals appear to be food generalists rather than specialists. As general grazers they tend to switch food sources and ingest dead organic matter, consisting not only of plant detritus but also of bacteria, fungi, algae, protozoa and nematodes; also intraguild predation and cannibalism is widespread in decomposer food webs (Scheu and Setälä, 2001). Additionally, most of what is ingested is egested, and only little is assimilated, so the actual resources used by detritivores often remain obscure (Scheu, 2005; Scheu et al., 2005). The fact that predators in soil also have remained generalist feeders presumably is related to the high density of potential prey and the difficulties of locating specific prey in the opaque and porous soil habitat (Scheu and Falca, 2000; Ponsard and

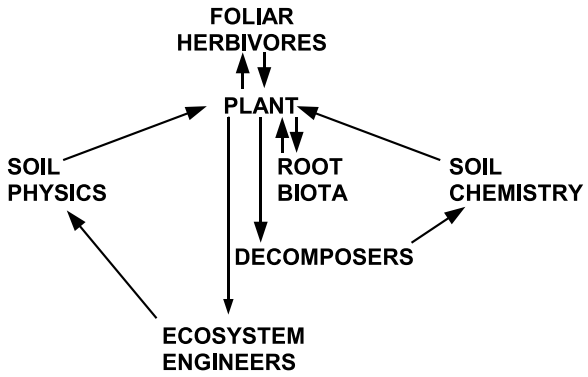


Figure 1.1:
Conceptual diagram of an interaction web, showing the main interactions between plants and biotic and abiotic soil constituents. (Brussaard, 1998)

Arditi, 2000; Maraun et al., 2003).

At a functional level soil organisms are often grouped in guilds, combining species with similarities in life-history tactics, microhabitat, principal food, feeding mode and ecophysiology (Brussaard, 1998). Roughly, soil organisms can be grouped into three guilds. The first group consists of root biota with close association to the living plant, which are either beneficially or detrimentally for plant growth. Second is the microflora, and the micro- and mesofauna acting as regulators of numbers and activities of microorganisms and microbial feeders. This group includes the decomposers. The third group consists of meso- and macrofauna, which creates microhabitats for other soil biota by reworking the soil, so called ecosystem engineers, of which earthworms and termites are the most important. Plants are affected by all three guilds and each of the guilds is affected by plants, as depicted in Fig. 1.1.

Moore et al. (1990), and Moore and de Ruiter (1991) proposed 5 trophic levels for a soil food web.

1. detritus and primary production (roots, algae)

2. primary decomposers and herbivores (bacteria, fungi, phytophagous nematodes)

3. consumers of bacteria and fungi (flagellates, mycophagous nematodes and mites, bacteriophagous nematodes and mites, Collembola)

4. intermediate predators (ciliates, predatory nematodes and nematophagous mites)

5. top predators (ciliates, amoebae, predatory mites)

This model is widespread in literature, but this classification of trophic levels is mainly based on taxonomic groups and therefore is only a rough approach to describe trophic relationships in soil.

1.2 Collembola in the decomposer food web

Collembola are among the most widespread and abundant terrestrial arthropods with up to several million individuals per square meter (Rusek, 1998). They occur from seashore to mountain tops, from tropical rainforest to the Antarctic. Worldwide nearly 7500 species have been described. Bodysize of Collembola is between 0.25 and 10 mm length. Euedaphic species are small and without pigment and live in the soil, epedaphic species are bigger and pigmented and occur near or above surface. Collembola occupy very different trophic niches and are known to feed on bacteria, nematodes, enchytraeids, fungi, litter, living plant parts and algae (Rusek, 1998; Fig. 1.2). Fungi are regarded as the most important food source (Visser and Whittaker, 1987; Chen et al., 1995; Klironomos and Kendrick, 1995). Chahartaghi et al. (2005) grouped Collembola into 3 feeding guilds based on stable isotope analysis:

1. phytophages/herbivores: feeding mainly on lichens, algae and plant tissue
2. primary decomposers: feeding on litter detritus with adhering fungi and bacteria
3. secondary decomposers: predominantly feeding on microorganisms, in particular fungi, comprising a subgroup, which feeds also on other soil animals like nematoda, rotifers, protozoa and on eggs of other Collembola

Collembola play an important role in soil by forming soil microstructure in arctic, alpine and weakly developed soils in early succession stages. In more developed soils Collembola participate in the disintegration of leaf litter and in secondary disintegration of macro- and megafauna excrements (Rusek, 1998). They are hosts of many parasitic protozoa, nematoda, trematoda and pathogenic bacteria. They are food sources for many predators like carabid beetles and their larvae (Bauer, 1985), dipterans, ants (Hölldobler and Wilson, 1990), mites (Karg, 1971), other Collembola (Cassagnau, 1972), frogs, reptiles, pheasant chicks (Rusek, 1998) and spiders (Nyffeler, 1999; Fig. 1.2). Collembola are specialised feeders on soil microbiota and control the population dynamics of these organisms in soil (Parkinson, 1983). They have a strong and generally positive impact on N mineralisation, soil respiration, leaching of dissolved organic carbon and plant growth. Key mechanisms are fungal feeding, distribution of soil biota propagules (especially fungal propagules), root herbivory and predation on nematodes (Filser, 2002).

1.3 Methods to investigate soil food webs

Decomposer communities are extraordinary complex, with a huge amount of species on small space. Baseline of this food web is a uniform mixture of leaf litter, root litter and root exudates. Linkage between components of the food web is poorly understood (Scheu and Falca, 2000; Scheu, 2002). This is mainly due to the fact that trophic relationships in soil food webs are difficult to assess, because of the small size of the animals and the cryptic habitat.

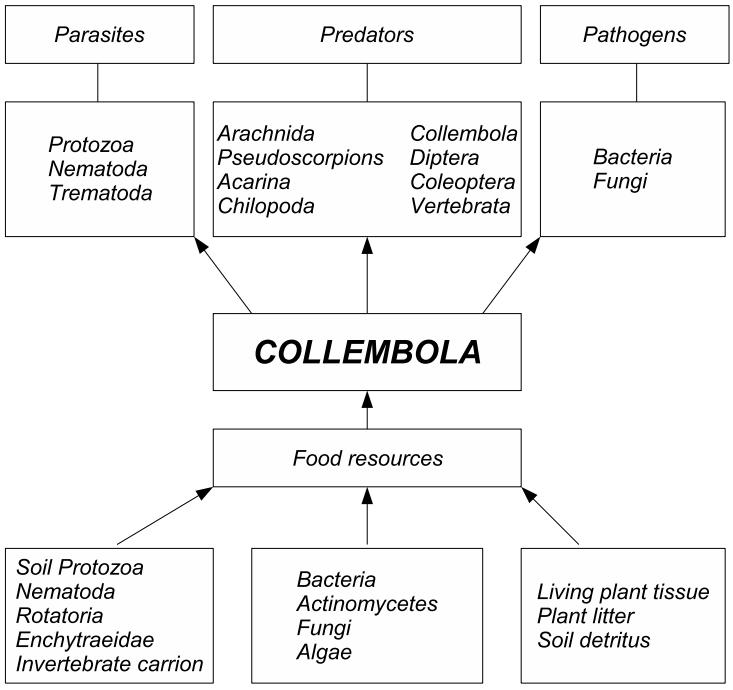


Figure 1.2:
Functional links of Collembola to other groups of organisms and food sources. (Rusek, 1998)

1.3.1 Classic approaches

First investigations on feeding strategies were made by direct observation or investigation of morphological characteristics. One approach to get insight into food preferences of Collembola is the comparison of mouthparts. Wolter (1963) differentiated between sucking and chewing mouthparts. Sucking Collembola feed mainly on bacteria, yeast and fluids from fermenting liquor and water films covering soil particles. Chewing Collembola feed on more solid food sources, like fungi, litter or algae. Christian (1989) found that co-existing intertidal Collembola equipped with different mouth structures feed on different food materials.

Chen et al. (1996) combined morphological criteria of mouthparts with gut content analysis by light microscopy. They found animals with large mandibles and labrum to ingest a greater proportion of large particles, whereas animals with the smallest mandibles and labrum ingest mostly small particles. Ponge (2000) distinguished species with different feeding habits by gut content analysis, but found that most species (onychiurid and isotomid species) ingest a wide spectrum of food material. However, food specialisation was assigned for *Friesea* spp. as a predator, and *Willemia* spp. as fungivore. A disadvantage of gut analysis is that some food items are digested very slowly, whereas others, like nematodes, are digested very fast and therefore are rarely detected by this method (Lee and Widden, 1996). Besides this, it is always only a snapshot of what the animal recently fed on and does not show the general variety of food material. These differences generally bias the results of gut content analyses (Sheppard and Harwood, 2005).

Feeding strategies of animals can also be assessed by investigation of fitness parameters

to test the suitability of certain food sources. This has been performed for nematodes reared on 17 different fungi (Ruess et al., 2000). Survival and development was different depending on fungal diet. Verhoef et al. (1988) tested the importance of green algae and fungi for Collembola. Feeding activity was estimated by counting the number of animals present on the food source. Growth was measured by weighing the Collembola, moulting rate was estimated by counting exuvia, and fecundity by counting the number of juveniles. Main result from this study and from Scheu and Simmerling (2004) was, that Collembola benefit from a mixed diet.

Another classical method to analyse food preferences is to perform food choice experiments. By using this approach several studies indicated that Collembola show a preference for certain fungi (Klironomos and Kendrick, 1995; Scheu and Simmerling, 2004) and that *Folsomia candida* select nematodes over fungi (Lee and Widden, 1996). Food choice experiments were also performed for nematodes (Ruess et al., 2000) suggesting preferential ingestion of mycorrhizal species (i.e. Basidiomycetes) over mitosporic forms. In contrast, oribatid mites rather ingested dark pigmented fungi than hyaline forms (Maraun et al., 1998).

Evaluation of food choice experiments can be complicated depending on the method used. Direct observation is difficult, because normal behaviour of animals may be altered due to disturbance with light and the movement of pots. Assessing the amount of consumed food or counting of faecal pellets is a rough estimate. To avoid these problems, Thiele (1990) used colour marked fungi as food source for choice experiment with Collembola, and detected the preferred fungi by the gut colour of the animals. The study revealed that Collembola choose between different fungi, and feed on one food source until they are full and then move on to the next diet.

1.3.2 Stable isotope analysis

An advanced technique to investigate the feeding behaviour of animals on a more long-term scale is stable isotope analysis. Naturally occurring stable isotopes of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) provide insight into dietary and trophic relationships within food webs (Gannes et al., 1997, 1998; Ponsard and Arditi, 2000; McCutchan et al., 2003). Animals higher in the food chain tend to be enriched in the heavier isotope compared to animals at lower trophic levels (Gannes et al., 1998; Minagawa and Wada, 1984). Natural isotope variation or fractionation depends on thermodynamic equilibria and kinetic processes affecting the individual isotope. In both cases, fractionation is a function of slight variation in physical and chemical properties of isotopes (Broecker and Oversley, 1976).

Generally, the fractionation between resource and consumer's tissue nitrogen is in the range of 2 - 5‰ and indicates the mean trophic level of an animal (DeNiro and Epstein, 1981; Post, 2002). In most cases a difference of 3.4‰ is used to group animals into trophic levels. The trophic level changes in $^{15}N/^{14}N$ ratios were used to get insight into the structure of soil food webs (Ponsard and Arditi, 2000; Scheu and Falca, 2000; Schmidt

et al., 2004; Schneider et al. 2004), including Collembola (Chahartaghi et al., 2005).

In contrast, the isotopic composition of carbon ($^{13}C/^{12}C$ ratio) of the whole body in animals closely resembles that of their diet, with an overall enrichment of about +1‰ per trophic level (DeNiro and Epstein, 1978; Post, 2002). Carbon isotope ratios therefore can be used to determine the food source of an animal. Plants differ distinctly in carbon isotopic composition depending on the photosynthetic pathway (C_3 or C_4) (DeNiro and Epstein, 1978; Post, 2002). Briones et al.,(1999a) investigated feeding preferences of *Folsomia candida* and *Proisotoma minuta* with two different naturally labelled diets (C_3 soil, C_4 litter) using carbon stable isotope analysis. They found a clear preference in new organic matter derived from maize litter. Further studies successfully used stable isotope techniques to assess feeding strategies of Collembola (Chamberlain et al., 2004; Scheu and Folger, 2004; Ruess et al., 2005a) and earthworms (Briones et al., 1999b, 2001; Briones and Bol, 2003).

Besides diet, other factors can affect the isotopic composition of an animal. Some studies revealed food deprivation and food quality to have important impact (Adams and Sterner, 2000; Hobson et al., 1993; Rothe and Gleixner, 2000; McCutchan et al., 2003; Pearson et al., 2003; Gannes et al., 1997). Also, the amount of fat deposit influences the isotopic carbon signal (Focken and Becker, 1998; Gearing, 1991). Generally, individual protein and fat balance can have a strong impact on the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ composition in animals (Gaye-Siessegger et al., 2004a,b). The various factors influencing stable isotope discrimination by animals involve some uncertainty in the use of this method for ecological research, and calls for defined laboratory experiments (Gannes et al., 1997).

1.3.3 Fatty acid analysis

Fatty acids (FAs) have been used as qualitative markers to trace or confirm predator-prey relationships in the marine environment for more than thirty years. Comparable to stable isotopes they provide information on the dietary intake over a longer period of time. Energetically it is more efficient to incorporate dietary FAs without modification into body tissue, which leads to a lipid pattern in the consumer that reflects the composition of its diet (Stott et al., 1997). The specificity of FA composition in different taxonomic groups is used as biochemical marker of trophic and metabolic interactions in aquatic food webs (Desvilettes et al., 1997; Leveille et al., 1997). FA markers were applied to map the transfer of carbon and to understand the feeding strategies of consumers (Ederinton et al., 1995; Gladyshev et al., 1999, 2000). Only recently FA analysis has been suggested as a promising tool to investigate trophic interactions in soil. Ruess et al. (2002, 2004, 2005a) and Chamberlain et al. (2004) reported trophic transfer of FAs from the fungal food source to nematode and Collembola grazers. Lipid pattern gave information on the food source of animals and FA biomarkers for different feeding strategies have been assigned (Chamberlain et al., 2005; Ruess et al., 2005b).

1.3.4 Compound-specific fatty acid analysis

The combination of stable isotope and fatty acid analysis to a compound-specific monitoring of $^{13}C/^{12}C$ ratios in fatty acids with gas-chromatography-combustion-isotope-ratio-monitoring-mass-spectrometry (GC-C-IRM-MS) offers new insight into belowground food webs. This approach was used to distinguish chemosynthetic (based on chemoautotroph bacteria) and photosynthetic (based on photosynthetic plants) food chains in marine systems (MacAvoy et al., 2002; 2003). In soils the $^{13}C/^{12}C$ ratios in FAs can be used to identify bacterial substrates and the flux of carbon in microbial communities (Abraham et al., 1998). Arao (1999) used the incorporation from ^{13}C-acetate into soil PLFAs to detect changes in soil bacterial and fungal activities. Boschker and Middelburg (2002) concluded in their review, that compound-specific analysis of biomarkers can help to include microorganisms into food web studies. Recent laboratory experiments on Collembola and nematodes have investigated the power of this method for soil food webs (Chamberlain et al., 2004; Ruess et al., 2005a). FAs which are synthesised de novo reflect the average $^{13}C/^{12}C$ ratio of food resources, whereas FAs with dietary routing reflect the $^{13}C/^{12}C$ ratio of the FAs of the food source. This can be used to verify the trophic transfer from a potential food source to consumers.

1.3.5 Further methods

There are further methods for analysing trophic interrelationships in food webs, which will not be discussed specifically in this work. Immunological techniques use the specifity of antibodies to detect an antigen. Antibodies, which bind to proteins of the potential diet of an animal, were used to identify the diet in the gut of the consumer. The binding is made visible by linking an enzyme to the antibody, which turns over a substrate into a coloured product. Or the antibody is linked to fluorescent substances. Immuno assays and ELISA (enzyme linked immuno-sorbent assay) techniques have been used to trace food relationships e.g. of beetles (Bohan et al, 2000) and other arthropods (Krautz et al., 2003). FISH (fluorescence in situ hybridisation) was applied to detect food material in the gut of earthworms (Fischer et al., 1995; 1997).

Another approach was used by Berg et al. (2004) by investigating the enzymatic activity of carbohydrases in the gut of Collembola and grouping them into feeding guilds by this physiological criteria.

DNA based techniques use the ability to differentiate between unique pieces of DNA from predator and prey species. Approaches with a polymerase chain reaction (PCR) step to amplify dietary DNA are highly effective even on carrion prey (Juen and Traugott, 2005). Although this technique is thought to replace the immunological techniques (Symondson, 2002), these can sometimes still be more advantageous in field assessment of predation (Sheppard and Harwood, 2005). Proteins can be detected much longer in the gut of a consumer (Harwood et al., 2001; Schenk and Bacher, 2004), than prey DNA (Agusti et al., 2003; Sheppard et al., 2004). Also the specifity of the antigen can be adapted to the taxonomic level which is investigated, i.e. antigens can be specific for one

prey species or for a whole family (Symondson et al., 1999). Either way a disadvantage of gut content analysis by DNA or immunological techniques and in general is that one may see what the animal feed on, but not which parts they really absorb and use as energy resource.

1.4 Aim of this study

In a first step I wanted to investigate the reliability of stable isotope signals for soil food web analysis and considered factors which can influence the obtained results. Food depletion (i.e. starvation), food quality (i.e. C/N ratio of fungal diet) and life stage are expected to affect the fractionation of ^{15}N and ^{13}C in Collembola, which may result in patterns not related to the consumed diet. Food quality likely will alter consumption rates and nutritional status in Collembola. Starvation strongly affects their metabolism by changing the ratio of anabolic to catabolic processes, and similarly different life stages alter the balance between metabolic pathways.

Further insight into trophic relationships in soil was gained by the analysis of the fatty acid composition of consumers and their diet. The lipid composition of animals is not fixed and in particular their diet may exert strong influences on the shape of FA profiles. To use FAs as biomarkers in food webs, the factors affecting their trophic transfer and composition need to be analysed. The influence of starvation, food quality, developmental stage and temperature on the FA composition of Collembola was investigated. In another experiment specific FA markers for bacterial consumption in Collembola were evaluated.

Finally, stable isotope and fatty acid analysis was combined in a compound-specific analysis and applied to an agricultural food web in the field. $^{13}C/^{12}C$ ratios of soil PLFAs, Collembola and spider FAs were determined and used to assess trophic interactions.

Chapter 2

Effects of fungal food quality and starvation on the fatty acid composition of *Protaphorura fimata* (Collembola)

Abstract

The lipid pattern of animals is influenced by species, life stage, environmental conditions and diet. We investigated the effects of food quality and starvation on the phospholipid (PLFA) and neutral lipid (NLFA) fatty acid pattern of the Collembola *Protaphorura fimata*. Collembola were fed with two common soil fungi, *Agrocybe gibberosa* and *Chaetomium globosum*, of which the cellular lipid composition was analysed. *A. gibberosa* was grown on agar with different nitrogen content, resulting in altered fatty acid pattern and C/N ratio, i.e. fungi of different food quality. Collembola did not mirror the lipid composition of the fungal diet as the pattern of major NLFAs in *P. fimata* was vice versa. Presumably, altered food quality of fungi caused compensatory responses by the Collembola, thereby diminishing the fungal signal. In a further experiment *P. fimata* (previously maintained with *C. globosum*) was kept without food for up to four weeks. Starvation resulted in a decline in the total amount of NLFAs, however, it did not affect the fatty acid pattern, indicating that NLFAs were degraded indiscriminately. Generally, the PLFA profile of the Collembola changed only slightly due to variations in diet quality or starvation.

2.1 Introduction

Lipid composition of microorganisms and fungi is frequently used as a biomarker in environmental samples. Phospholipid fatty acids (PLFAs) are components of cell membranes and have been used to quantify and classify microorganisms in soil (Tunlid and White, 1992; Frostegård and Bååth, 1996; Zelles, 1999). Different groups of microorganisms synthesize specific fatty acids through various biochemical pathways, which makes PLFAs

effective taxonomic markers to define community composition (Bååth et al., 1998; Navarrete et al., 2000). The unsaturated fatty acids 18:2ω6,9 (linoleic acid) and 18:1ω9 (oleic acid) have been applied to estimate fungal biomass (Frostegård and Bååth, 1996; Mikola and Setälä, 1998). In contrast to membrane PLFAs, neutral lipid fatty acids (NLFAs) are predominantly storage lipids and often constitute a high percentage of the total lipid content in animals. Due to their role as energy reserves NLFAs are closely related to nutritional requirements and metabolism (Stanley-Samuelson et al., 1988). Recently, fatty acids were suggested as a tool to investigate soil food webs, where direct observation of animals is difficult because of their small size and their cryptic habitat. Ruess et al. (2002) reported for nematodes, that the lipid composition is controlled by both the nematode species and its diet and concluded that monitoring fatty acid patterns of soil animals may therefore provide an effective tool to detect trophic interactions in belowground food webs.

Collembola are widespread in soil systems and play an important role in decomposition processes (Visser, 1985). They are known to feed on soil microbiota, including bacteria, fungi, actinomycetes, and algae (Rusek, 1998). Several studies have demonstrated the importance of fungi in Collembola nutrition (Visser et al., 1987; Chen et al., 1995; Klironomos and Kendrick, 1995). Food selection in Collembola is influenced by physiological factors, such as the nutritional status of the fungal hyphae, or their age with low growing or senescent hyphae being less preferred (Booth and Anderson, 1979; Leonard, 1984). Differences in fungal food quality (e.g. nitrogen content, fungal host species, mixed diet) affect the reproduction and frequency of moulting in Collembola (Joosse and Testerink, 1977; Walsh and Bolger, 1990; Chen et al. 1995; Scheu and Simmerling, 2004). Starvation strongly affects the metabolism of Collembola. Generally, the lack of energy supply results in changes in body water content, respiration and reproduction (Hubert and Šustr, 2001). Also, food depletion negatively affects drought and cold tolerance of Collembola (Lavy et al., 1997).

Lipids, as stored triacylglycerols, play an essential role as reserve of metabolic energy, in addition to the function of phospholipids as membrane components (Canavoso et al., 1998). However, only few studies have investigated the fatty acid profiles of Collembola lipids (Stransky et al., 1986; Holmstrup et al., 2002). To use fatty acids as biomarkers in food webs the factors that affect their trophic transfer and pattern need to be analysed. Lipid composition is not fixed and in particular diet and development may excert strong influences on the shape of fatty acid profiles. The relative abundance of fatty acids in insects is determined by the specific type of biosynthetic pathway of the given species (Hanson et al., 1985; Ghioni et al., 1996), the life stage (Ogg and Stanley-Samuelson, 1992; Sayah et al, 1997), the environmental conditions (Joanisse and Storey, 1996) and the diet (Thompson et al., 1973; Fernando-Warnakulsuriya et al., 1988; Howard and Stanley-Samuelson, 1996). The composition of NLFAs in the fat body results from different processes including the storage of dietary lipids, de novo synthesis, degradation and subsequent release for mobilisation to sites where they are metabolised (Beenakkers et al., 1985). The pattern of PLFAs, which are structurally and functionally involved in

biomembranes, is individually arranged in specific tissues and controlled at the cellular level (Stanley-Samuelson et al., 1988).

The aim of our experiments was to investigate the influence of food quality and starvation on (i) the lipid content of Collembola and (ii) the pattern of neutral lipid and phospholipid fatty acids. Variations in dietary nutrient supply is a situation frequently faced by soil living animals and the results were expected to provide insight into the response in lipid metabolism of Collembola.

2.2 Materials and Methods

2.2.1 Fungi

Two soil fungal species were used as food for Collembola in the experiments: the ascomycete *Chaetomium globosum* Kunze and the basidiomycete *Agrocybe gibberosa* (Fr.) Fay. *C. globosum* was cultivated on Potato dextrose agar (PDA, Merck). *A. gibberosa* was grown on Pachlewska agar adjusted to different N concentrations in order to alter the C/N ratio. Pachlewska agar is a nutrient rich medium that contains 20 g glucose, 5 g maltose, 0.5 g $MgSO_4 \cdot 7\,H_2O$, 1 g KH_2PO_4, 50 μg thiamine HCl, 0.5 ml of 1% w/v Fe citrate, 0.5 ml of 2% w/v $ZnSO_4$ and 15 g agar per 1 l distilled water. As nitrogen source we added 0.5, 0.75, 1.5 and 3 g NH_4 tartrate per 1 l (equivalent to 75, 150, 300, 600 μg N/g agar). For the analysis of fatty acids, *A. gibberosa* was grown in liquid cultures of the same media and *C. globosum* was grown on PDA covered by a membrane filter (Millipore, 0.8 μm). Fungi were kept at 15°C (three replicates per treatment).

2.2.2 Collembola

Collembola of the species *Protaphorura fimata* (Gisin, 1952) were taken from laboratory cultures fed with bakers yeast. Specimens were put into plastic microcosms (diameter 7 cm, height 4.5 cm) containing a layer of plaster mixed with activated charcoal (2:1) at the bottom. Microcosms were kept at 15°C in darkness and kept moist with distilled water. Each microcosm contained 10 individuals of *P. fimata*. This low density was chosen to avoid cannibalism due to crowding. Eggs, pellets and exuvia were removed once a week. Collembola were fed with fungal diet ad libitum. Round pieces of fungal mats with a diameter of 10 mm were cut out of the agar cultures under sterile conditions, put into the microcosms and were renewed once a week.

2.2.3 Experimental set-up

In the first experiment the effect of starvation on the fatty acid pattern of *P. fimata* was investigated. The Collembola were fed for six weeks with *C. globosum*. Then the fungus was removed and animals were kept four weeks without food supply. Specimens were sampled at the beginning (day 0), after 2, 4, 8, 14, 21 and 28 days. The second

experiment investigated changes in the fatty acid pattern due to different food quality represented by the different C/N ratio of the agar used as culture media for the fungus (75, 150, 300 or 600 μg N/g agar). *P. fimata* was fed with *A. gibberosa* ad libitum for six weeks. For both experiments three replicates per treatment were performed and the Collembola were frozen at -20°C until analysis.

2.2.4 Analysis of fatty acid patterns

Cellular lipids of Collembola harvested from the microcosms were divided into different lipid classes using a method described in Zelles (1999). Collembola biomass was extracted by shaking in 5 ml single phase extraction solvent (chloroform/methanol/0.05 M phosphate buffer (pH 7.4) 1:2:0.8) overnight. The solvent was then transferred to new tubes and samples were re-extracted by shaking for 2-3 h with additional 2.5 ml. Extraction solvents of both steps were combined, 0.8 ml distilled water and 0.8 ml $CHCl_3$ were added and samples centrifuged at 1500 rpm for 5 min. Samples were allowed to stand and separate. Then the top two phases were removed and the chloroform fraction of each sample was transferred to a silica acid column (0.5 g silicic acid, mesh size 100-200 μm). Lipids were eluted with 5 ml chloroform (neutral lipids), 8 ml acetone (glycolipids) and 5 ml methanol (phospholipids). Neutral lipids and phospholipids were used for data analysis. Separation of these two lipid classes is suggested to be adequate, as silica acid columns were washed with acetone for glycolipids inbetween elution of the two fractions. Frostegård et al. (1991) report a 85% recovery of the total phosphate in the eluates of the methanol fractions and suggest the method for extraction and digestion to give reliable results. The chloroform and methanol fraction was reduced by evaporation (50°C, vacuum 200 hPa) in a Labconco RapidVap.

Fungal biomass of *A. gibberosa* was harvested from liquid cultures (Pachlewska media). For *C. globosum* membrane filters were stripped of the agar with the adhering fungal mats and hyphae were harvested by scraping from the filter with a sterile scalpel. Chloroform and methanol fractions of the Collembola samples and total fungal biomass were saponified and methylated following the procedures given for the Sherlock Microbial Identification System (MIDI Inc., Newark, Del.). Saponification of lipids was conducted in a sodium hydroxide-methanol solution (45 g sodium hydroxide, 150 ml methanol, 150 ml distilled water) at 100°C for 30 min, followed by acid methanolysis in HCl-methanol (325 ml 6.0N hydrochloric acid, 275 ml methyl alcohol) at 80°C for 10 min. The fatty acid methylesters were extracted into hexane-methyl tertiary butyl ether (1:1) and washed with aqueous NaOH (10.8 g sodium hydroxide, 900 ml distilled water). The lipid-containing phase was then transferred to test tubes and stored at -20°C until analysis.

Fatty acid methyl esters were analyzed by gas chromatography (GC) using the Sherlock Microbial Identification System (MIDI Inc.) consisting of a Hewlett Packard 5890 Series II gas chromatograph and flame ionization detector equipped with an HP Ultra 2 phenyl methyl silicone fused capillary column (25 m $\times$ 0.2 mm i.d., film thickness 0.33 μm), an automated sampler and computer with associated software (Sherlock Pattern Recognition

Software, MIDI). The fatty acid methyl esters were identified on the basis of their retention times and quantified. To verify correct identification of fatty acids methyl esters (chain length and saturation) a range of samples containing NLFAs and all samples with PLFAs were analyzed by GC-mass spectrometry using an Agilent Series 6890 GC System and 5973 Mass Selective Detector, equipped with a HP5MS capillary column (30 m $\times$ 0.25 mm i.d., film thickness 0.25 μm). Generally, the relative amounts of identified fatty acids were calculated according to data from GC analysis using the Microbial Identification System (MIDI). GC mass spectrometry was applied to clarify correct identification of fatty acids. If biomass in samples was low, i.e. for most PLFA fractions, the combined data obtained with the two instruments were used. The fluidity of lipids was expressed as the fatty acid unsaturation index (UI). This was calculated as follows:

$$UI = \frac{(\mathrm{C{:}1} \cdot 1) + (\mathrm{C{:}2} \cdot 2) + (\mathrm{C{:}3} \cdot 3) + (\mathrm{C{:}4} \cdot 4)}{100}$$

where C:1, C:2, C:3 and C:4 represents the proportion (%) of fatty acids with 1, 2, 3, and 4 double bonds, respectively.

2.2.5 C/N analysis

To determine the C/N ratio of the fungi dried samples were weighed into tin capsules and total C and N content was measured by an elemental analyser (NA 1500, Carlo Erba, Milan).

2.2.6 Statistical analysis

Differences in fatty acid profiles of fungi and Collembola were analysed using ANOVA. If significant effects were suggested, differences between means were analysed by Tukey's honestly significant difference test. Statistical analyses were performed using SAS (SAS Institute Inc., Cary, USA).

2.3 Results

2.3.1 Fatty acid pattern

Total cellular lipids of *A. gibberosa* contained 10 predominant FAs with a carbon chain length ranging from 12 to 18 (Table 2.1). The major saturated fatty acid was palmitic acid (16:0) with 18.4 - 39.3% of total FAs. Linoleic acid (18:2ω6,9) occurred with 47.4 - 76.1%. Concentrations of 12:0, 14:0, 15:0, 17:0, 17:1ω8, 17:1ω9, 18:0, and 18:1ω9 were low. *C. globosum* contained 7 predominant FAs with a carbon chain length ranging from 15 to 18 (data not presented). The major saturated fatty acid was palmitic acid with 15% of total FAs. The most abundant monoenoic fatty acid was oleic acid with 22.7% and the only polyenoic fatty acid was linoleic acid with 56.7% of total FAs. Proportions of 15:0, 17:0, 18:0 and 17:1ω9 were low (altogether 5.6%).

Fatty acid composition	μg N/g agar 75	150	300	600
Saturated				
12:0	0.7 ±0.9	0.7 ±0.7	0.2 ±0.4	0.2 ±0.4
14:0	1.1 ±0.2	0.7 ±0.1	0.7 ±0.1	0.7 ±0.1
15:0	2.8 ±0.5 a	1.3 ±0.4 b	0.8 ±0.1 b	0.8 ±0.1 b
16:0	**39.3 ±7.1 a**	**20.1 ±1.3 b**	**19.7 ±1.3** b	**18.4 ±2.6 b**
17:0	1.2 ±0.1 a	0.4 ±0.4 b	0.2 ±0.3 b	0 b
18:0	4.2 ±0.9 a	2.4 ±0.5 b	1.5 ±0.2 b	1.3 ±0.4 b
monoenoic				
17:1ω8	0 b	0.7 ±0.2 a	0.2 ±0.3 ab	0.2 ±0.3 ab
17:1ω9	0.6 ±0.6	0	0.2 ±0.3	0
18:1ω9	2.7 ±0.7	3.4 ±1.3	2.2 ±0.9	2.3 ±0.8
Polyenoic				
18:2ω6,9	**47.4 ±8.1 b**	**70.3 ±2.4 a**	**74.5 ±1.8 a**	**76.1 ±2.2 a**
C16:C18	0.75 ±0.22 a	0.26 ±0.02 b	0.25 ±0.02 b	0.23 ±0.04 b
Unsaturation Index	0.98 ±0.16 b	1.45 ±0.04 a	1.51 ±0.02 a	1.55 ±0.05 a

Table 2.1:
Cellular lipids (fatty acids in % ± s.d.) of the fungus *A. gibberosa* grown on Pachlewska agar with 75, 150, 300 or 600 μg N/g agar. Major FAs in bold. Means within rows sharing the same or no letters are not significantly different from each other (Tukey's HSD, $P < 0.05$)

Cellular fatty acids of Collembola were divided into neutral lipids (NLFAs) and phospholipids (PLFAs). Generally, the Collembola contained higher number of FAs than their diet. *P. fimata* fed ad libitum with *C. globosum* contained 13 NLFAs with a carbon chain length ranging from 14 to 20 (Table 2.2). The major saturated fatty acid was palmitic acid with 23.5% of total NLFAs. Oleic acid was the most abundant monoenoic fatty acid (34.3%) and linoleic acid the most abundant polyenoic fatty acid (27.4%). The remaining 14.8% of NLFAs consisted of 14:0, 15:0, 17:0, 18:0, 16:1ω7, 17:1ω8, 17:1ω9, 18:3ω6,9,12, 20:2ω6,9 and 20:4ω6,9,12,15. Several long-chain polyenoic FAs occurred, in particular with 20 C such as arachidonic acid (20:4ω6,9,12,15). The PLFA pattern of *P. fimata* fed ad libitum with *C. globosum* consisted of 11 FAs with a carbon chain length ranging from 12 to 20 (Table 2.3). The major saturated acids were palmitic acid and stearic acid with 20.4% and 22.7% of total PLFAs, respectively. Oleic acid was the most abundant monoenoic fatty acid (21.1%) and linoleic acid the most abundant polyenoic fatty acid (22.4%). The remaining 13.4% consisted of 12:0, 14:0, 15:0, 17:0, 20:1ω6, 20:3ω6,9,12, 20:4ω6,9,12,15.

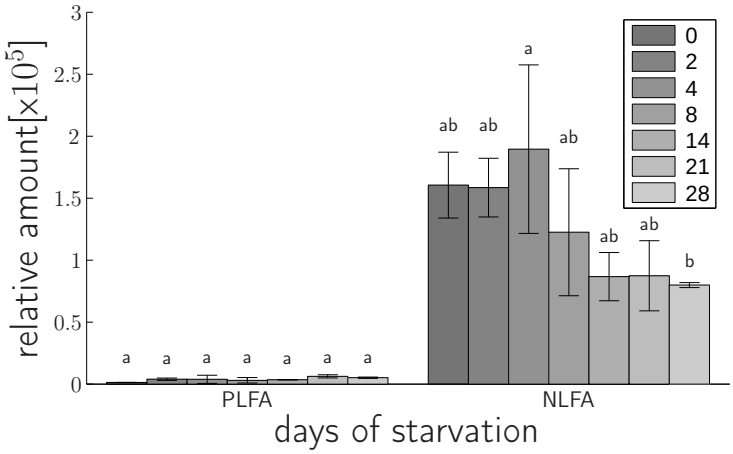


Figure 2.1:
Relative amount of PLFAs and NLFAs per g dry weight of the Collembola *P. fimata* starved for up to 28 days. Bars (± s.d.) sharing the same letters are not significantly different (Tukey's HSD, $P<0.05$).

2.3.2 Starvation

Starvation of *P. fimata* for four weeks affected the body mass and water content of the Collembola. Fresh and dry weight were significantly affected ($F_{6,14}$=10.17, P=0.0002; $F_{6,14}$=5.19, P=0.0053, respectively) and ranged between 83-243 and 27-54 mg/ind, respectively (data not presented). Fresh weight was 189 mg/ind at day 0 and, except of a significant decrease to 44% of the initial weight on day 8, remained at a similar level until the end of the experiment. Dry weight of ad libitum fed Collembola was 54 mg/ind (day 0). It was reduced to 64% of initial weight within the first 2 days and decreased further to 51% after 14 days, thereafter it remained almost constant.

During starvation the total amount of NLFAs declined significantly ($F_{6,13}$=3.84, P=0.0200), whereas the amount of PLFAs was unchanged ($F_{6,8}$=1.49, P=0.2922, Fig. 2.1). A starvation period of 28 days did not significantly affect the three most abundant NLFAs (palmitic, oleic and linoleic acid; $F_{6,14}$=0.95, P=0.4887; $F_{6,14}$=1.04, P=0.4384; $F_{6,14}$=0.95, P=0.4889, respectively) of the Collembola (Table 2.4). However, some of the less frequent NLFAs were affected by starvation. Palmitoleic acid (16:1ω7) on day 0 had a significant lower proportion then on day 21 ($F_{6,14}$=3.70, P=0.0205) and stearic acid (18:0) significantly increased during the first 14 days ($F_{6,14}$=5.07, P=0.0059). Starvation did not affect the C16/C18 ratio of the fatty acids ($F_{6,14}$=1.02, P=0.4536) and there was no change in the Unsaturation Index (*UI*) ($F_{6,14}$=0.88, P=0.5328, Table 2.2). Within PLFAs oleic acid was the only acid, which significantly decreased during starvation (Table 2.3, $F_{6,12}$=6.92, P=0.0023). The long-chain polyunsaturated PLFAs 20:3ω6,9,12 and 20:4ω6,9,12,15 increased ($F_{3,7}$=5.85, P=0.0254; $F_{3,7}$=3.32, P=0.0864, respectively) between day 8 and 28 with food depletion. Starvation did not significantly affect the C16/C18 ratio ($F_{6,12}$=1.82, P=0.18) and the *UI* ($F_{6,12}$=1.84, P=0.17).

Fatty acid composition	starvation period 0	2	4	8	14	21	28
Saturated							
12:0	0	0.7 ±0.7	0	0	0	0	0
14:0	0.5 ±0.4 ab	0.9 ±0.1 a	0.7 ±0.1 ab	0.8 ±0.2 ab	0 b	0.5 ±0.4 ab	0.6±0.5 ab
15:0	1.8 ±0.1	2.1 ±0.2	1.7 ±0.1	1.7 ±0.3	2.2 ±0.5	2.2 ±0.1	1.9±0.3
16:0	**23.5 ±0.4**	**23.7 ±0.3**	**22.8 ±0.8**	**23.0 ±1.7**	**24.1 ±2.5**	**23.0 ±1.0**	**21.8±0.8**
17:0	1.1 ±0.1	1.2 ±0.1	1.2 ±0.2	1.3 ±0.2	1.7 ±0.3	1.0 ±0.8	1.5±0.3
18:0	4.7 ±0.2 c	5.2 ±0.6 bc	5.8 ±0.2 abc	6.9 ±0.7 a	6.6 ±0.8 ab	6.1 ±0.7 abc	6.1±0.5 abc
20:0	0 b	0.2 ±0.3 ab	0.5 ±0.4 ab	0.8 ±0 a	0 b	0 b	0 b
monoenoic							
16:1ω7	3.3 ±0.2 b	3.8 ±0.4 ab	4.0 ±0.7 ab	4.0 ±0.3 ab	4.5 ±1.0 ab	5.4 ±0.9 a	5.2±0.8 ab
17:1ω8	0.2 ±0.4	0.7 ±0	0.7 ±0	0.5 ±0.5	0.8 ±0.8	1.1 ±1.0	1.0±0.9
17:1ω9	0.2 ±0.4	0	0	0	0	0	0
18:1ω9	**34.3 ±0.2**	**32.1 ±1.5**	**33.2 ±1.1**	**31.8 ±2.3**	**32.1 ±1.2**	**32.4 ±0.5**	**32.2±2.2**
Polyenoic							
18:2ω6,9	**27.4 ±1.0**	**26.5 ±0.6**	**26.3 ±2.1**	**25.6 ±0.9**	**25.2 ±1.6**	**25.7 ±1.3**	**26.2±0.8**
18:3ω6,9,12	1.1 ±0.1	1.3 ±0.3	1.1 ±0.3	1.7 ±1.0	1.2 ±0.2	0.8 ±0.7	1.1±0.1
20:2ω6,9	0.2 ±0.3	0	0.5 ±0.4	0.3 ±0.5	0	0	0.2±0.4
20:4ω6,9,12,15	1.8 ±0.3	1.7 ±0.2	1.6 ±0.2	1.7 ±0.5	1.7 ±0.4	2.0 ±0.2	2.3±0.3
C16:C18	0.43 ±0.01	0.46 ±0.02	0.44 ±0.03	0.46 ±0.03	0.49 ±0.05	0.48 ±0.02	0.45±0.02
Unsaturation Index	1.03 ±0.02	1.00 ±0.01	1.01 ±0.04	1.00 ±0.05	0.98 ±0.06	1.01 ±0.02	1.03±0.02

Table 2.2:

NLFA pattern (fatty acids in % ± s.d.) of the Collembola *P. fimata* starved for up to 28 days. Major FAs in bold. Means within rows sharing the same or no letters are not significantly different from each other (Tukey's HSD, $P<0.05$)

Fatty acid composition	starvation period						
	0	2	4	8	14	21	28
Saturated							
12:0	0.1 ±0.2	0	0.5 ±0.9	0	0	0	0
14:0	1.0 ±1.0	0.8 ±1.1	1.3 ±0.5	0.6 ±0.5	0.9 ±0.9	0.6 ±0.3	0.4±0.5
15:0	0.5 ±0.9	1.3 ±1.8	0.3 ±0.5	0	0	0.1 ±0.2	0
16:0	**20.4 ±4.3**	**20.2 ±0.5**	**15.7 ±3.7**	**20.2 ±2.0**	**20.1 ±5.1**	**17.6 ±1.9**	**14.8±0.0**
17:0	2.1 ±2.1	1.7 ±2.3	2.6 ±2.2	2.2 ±2.5	2.6 ±0.3	2.3 ±0.3	3.1±0.4
18:0	**22.7 ±4.2**	**25.9 ±1.8**	**26.0 ±2.5**	**27.6 ±7.5**	**27.2 ±1.6**	**22.3 ±3.0**	**20.4±1.6**
monoenoic							
18:1ω9	**21.1 ±3.9 a**	**20.0 ±2.5 ab**	**16.0 ±0.6 ab**	**21.1 ±3.3 a**	**12.8 ±1.4 b**	**13.2 ±0.0 b**	**17.2 ±0.7 ab**
20:1ω6	1.2 ±2.2	0.7 ±1.0	1.1 ±1.9	0	0.8 ±0.7	0.5 ±0.5	0
Polyenoic							
18:2ω6,9	**22.4 ±3.6**	**19.1 ±3.0**	**28.0 ±4.0**	**24.0 ±5.7**	**27.5 ±2.1**	**31.2 ±4.4**	**32.3±0.7**
20:2ω6,9	0	0	0	0	0	0.3 ±0.5	0
20:3ω6,9,12	1.2 ±2.2	0.7 ±1.0	2.3 ±0.5	0.6 ±1.0	1.6 ±1.4	3.2 ±0.4	3.7±0.1
20:4ω,9,12,15	7.2 ±4.5	9.7 ±6.4	6.3 ±0.4	3.8 ±3.3	6.5 ±2.0	8.7 ±0.5	8.2±0.8
C16:C18	0.31 ±0.03	0.31 ±0.01	0.28 ±0.06	0.28 ±0.01	0.30 ±0.08	0.26 ±0.03	0.21±0
Unsaturation Index	1.00 ±0.18	1.00 ±0.18	1.05 ±0.11	0.86 ±0.23	1.00 ±0.18	1.21 ±0.11	1.26±0.01

Table 2.3:
PLFA pattern (fatty acids in % ± s.d.) of the Collembola *P. fimata* starved for up to 28 days. Major FAs in bold. Means within rows sharing the same or no letters are not significantly different from each other (Tukey's HSD, $P<0.05$)

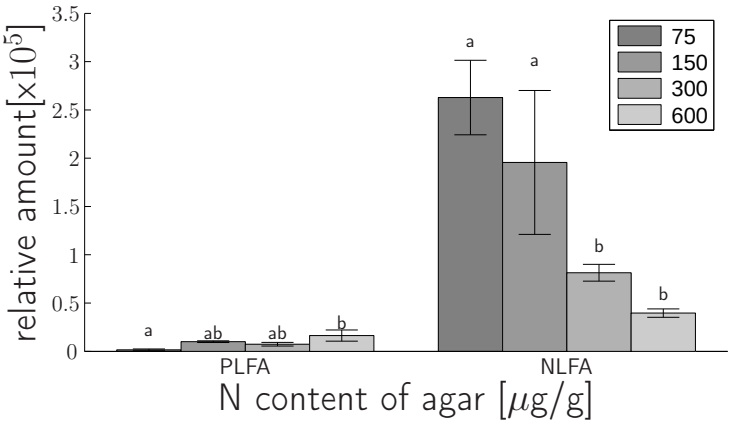


Figure 2.2:
Relative amount of PLFAs and NLFAs per g dry weight of the Collembola *P. fimata* reared on the fungus *A. gibberosa* grown on agar media with 75, 150, 300 or 600 μg N/g agar. Bars (± s.d.) sharing the same letters are not significantly different (Tukey's HSD, $P<0.05$).

2.3.3 N content

Concentrations of 75 and 150 μg N/g agar resulted in a significantly higher C/N ratio of *A. gibberosa* (16 and 19, respectively) compared to 300 and 600 μg N/g agar, where the C/N ratio was 9 ($F_{3,8}$=36.70, $P<0.0001$). Also, the fatty acid pattern of the fungus *A. gibberosa* grown on agar with 75 μg N/g agar had a higher proportion of palmitic acid ($F_{3,8}$=20.07, P=0.0004) and a lower content of linoleic acid ($F_{3,8}$=27.03,P=0.0002) compared to fungi grown on agar with higher N contents (Table 2.1). Linoleic acid generally increased and palmitic acid generally decreased with increasing N concentrations. Less frequent fatty acids also significantly differed with a decline in 15:0, 17:0 and 18:0 with higher N concentration in the agar ($F_{3,8}$=23.90, P=0.0002; $F_{3,8}$=16.01, P=0.0010; $F_{3,8}$=17.01, P=0.0008; respectively). Additionally, the *UI* increased from 0.98 to 1.55 and C16/C18 ratio decreased from 0.75 to 0.23 ($F_{3,8}$=27.60, $P<0.0001$; $F_{3,8}$=14.35, P=0.0014; respectively).

P. fimata fed with *A. gibberosa* had a fresh weight between 186 to 253 mg/ind and dry weight from 29 to 51 mg/ind. The N content of the fungus did not affect the Collembola fresh weight ($F_{3,8}$=0.84, P=0.5086). However, the dry weight at intermediate N concentrations (150 and 300 μg N/g agar) was significantly higher than on high and low N concentrations ($F_{3,8}$=28.94, P=0.0001).

The N content of the fungus significantly affected the relative amount of both NLFAs and PLFAs of Collembola. With increasing N concentrations of the food the amount of PLFAs increased and the amount of NLFAs decreased ($F_{3,7}$=10.22, P=0.0060; $F_{3,7}$=17.72, P=0.0007; respectively; Fig. 2.2). The relative amount of PLFAs increased 11 times and that of NLFAs was reduced by 85% in animals fed with fungi with high N content, compared to the Collembola reared with fungi with low N content.

Among the neutral lipids the proportion of palmitic acid significantly increased ($F_{3,8}$= 46.35, $P<0.0001$) and linoleic acid decreased ($F_{3,8}$=17.63, P=0.0002) with higher N con-

Fatty acid composition	μg N/g agar 75	150	300	600
Saturated				
12:0	0.2 ±0.3	0.5 ±0.5	0.5 ±0.5	0.7 ±1.2
14:0	0.6 ±0.2 b	0.4 ±0.4 b	0.7 ±0.6 b	2.1 ±0.1 a
15:0	2.4 ±0.1 b	2.0 ±0.2 b	2.3 ±0.1 b	4.2 ±0.6 a
16:0	**20.7 ±0.7 c**	**22.7 ±1.1 bc**	**24.6 ±0.2 b**	**30.6 ±1.8 a**
17:0	0.9 ±0.1	0.7 ±0.1	0.6 ±0.6	0.7 ±1.2
18:0	4.6 ±0.4 b	4.4 ±0.6 b	6.3 ±0.2 a	7.3 ±0.6 a
20:0	0.8 ±0.2	0.8 ±0.1	0.8 ±0.7 0	
monoenoic				
16:1ω7	3.4 ±0.7	4.5 ±0.4	6.6 ±0.4	5.5 ±5.1
17:1ω8	0.9 ±0.4 a	0 b	0 b	0 b
18:1ω9	**35.0 ±1.4** b	**43.7 ±1.0 a**	**40.7 ±0.4 a**	**29.1 ±2.7 c**
Polyenoic				
18:2ω6,9	**26.4 ±1.7 a**	**16.8 ±0.9 b**	**13.9 ±1.7 b**	**18.3 ±2.7 b**
18:3ω6,9,12	1.1 ±0.2 a	0.9 ±0.2 a	1.2 ±0.3 a	0 b
20:2ω6,9	0.9 ±0.1 a	0.8 ±0.1 a	0 b	0 b
20:4ω6,9,12,15	2.2 ±0.2	1.8 ±0.3	1.7 ±0.2	1.5 ±1.3
C16:C18	0.36 ±0.02 b	0.41 ±0.02 b	0.50 ±0.02 ab	0.66 ±0.16 a
Unsaturation Index	1.06 ±0.04 a	0.93 ±0.02 b	0.86 ±0.05 bc	0.77 ±0.02 c

Table 2.4:
NLFA pattern (fatty acids in % ± s.d.) of the Collembola *P. fimata* reared on the fungus *A. gibberosa* grown on Pachlewska agar with 75, 150, 300 or 600 μg N/g agar. Major FAs in bold. Means within rows sharing the same or no letters are not significantly different from each other (Tukey's HSD, $P<0.05$)

Fatty acid composition	μg N/g agar 75	150	300	600
Saturated				
12:0	2.5 ±1.7 a	0.1 ±0.3 b	0 b	0.3 ±0.4 b
14:0	2.1 ±2.2	1.73 ±0.6	1.7 ±1.6	1.5 ±0.2
15:0	1.5 ±2.3	1.4 ±1.0	0.8 ±0.7	1.8 ±0.1
16:0	**29.2 ±5.6**	**26.2 ±9.5**	**18.2 ±5.0**	**18.4 ±0.3**
17:0	3.6 ±5.3	2.5 ±0.3	2.8 ±1.0	5.1 ±0.1
18:0	**28.5 ±5.3**	**20.6 ±5.8**	**20.8 ±1.5**	**20.2 ±0.1**
monoenoic				
18:1ω9	**13.6 ±1.1**	**18.5 ±4.6**	**17.8 ±3.6**	**15.6 ±0.4**
20:1ω6	0	0	0	3.0 ±2.1
Polyenoic				
18:2ω6,9	**15.6 ±9.6**	**21.7 ±3.2**	**20.8 ±3.6**	**23.2 ±1.4**
20:2ω6,9	0	0	3.2 ±3.1	0
20:3ω6,9,12	0.6 ±1.0	2.4 ±2.3	4.7 ±1.3	3.5 ±0.2
20:4ω6,9,12,15	1.7 ±2.9	4.7 ±4.5	9.2 ±2.2	9.0 ±0.8
C16:C18	0.53 ±0.21	0.44 ±0.19	0.32 ±0.13	0.31 ±0.02
Unsaturation Index	0.53 ±0.31 a	0.88 ±0.25 ab	1.17 ±0.16 b	1.10 ±0.05 ab

Table 2.5:
PLFA pattern (fatty acids in % ± s.d.) of the Collembola *P. fimata* reared on the fungus *A. gibberosa* grown on Pachlewska agar with 75, 150, 300 or 600 μg N/g agar. Major FAs in bold. Means within rows sharing the same or no letters are not significantly different from each other (Tukey's HSD, $P<0.05$)

tent of the fungal food source (Table 2.4). The proportion of oleic acid was significantly higher ($F_{3,8}$=47.75, $P<0.0001$) at intermediate N levels (150 and 300 μg N/g agar). The C16/C18 ratio significantly increased from 0.36 to 0.66, whereas the *UI* decreased from 1.06 to 0.77 ($F_{3,8}$=7.87, P=0.0090; $F_{3,8}$=35.44, $P<0.0001$; respectively). Changes in PLFAs were less prevalent (Table 2.5). However, the *UI* increased significantly from 0.53 to 1.10 ($F_{3,7}$=4.52, P=0.046) with increasing N content of the food source.

2.4 Discussion

2.4.1 Fungi

The lipid composition of *C. globosum* and *A. gibberosa* as analysed in this study was similar to that of other fungal species (Stahl and Klug, 1996; Ruess et al., 2002). The major cellular fatty acids in fungi are 16:0, 18:0, 18:1 and 18:2 (Sancholle and Dalpé, 1993), which were well represented in both species. Fungal PLFAs and whole cell fatty acids, regardless of culture media, do not contain C20 (Weete, 1980; Johnk and Jones,

1994; Hering et al., 1999; Chen et al., 2001), as also observed in our analyses.

The lipid content of fungal species is variable and depends on growth conditions, species, stage of fungal development and age (Weete, 1980). In our experiment the fatty acid composition of *A. gibberosa* varied with the C/N ratio of the agar. The C/N ratio generally reflects the resource quality, with low C/N ratios being associated with high, and high C/N ratios with low quality (Weete, 1980). This likely affects fungal physiology, resulting in an altered metabolism and therefore in changes in fatty acid patterns. Low C/N ratios in the medium favor protein synthesis and high ratios favor lipid accumulation in fungi, i.e. fungi grown under nitrogen limiting conditions increase their lipid content (Weete, 1980). Our results indicate that this accumulation differs between fatty acids, as the fatty acid pattern changed with resource quality, which was also apparent in a decrease of the C16/C18 ratio and an increase of the unsaturation index. Additionally, the morphology of *A. gibberosa* was altered. Grown on 75 μg N/g agar, the fungus grew thin and rapidly over the medium, whereas with more N added, fungal mats were thicker and growth was slower.

2.4.2 Collembola

We found 15 different NLFAs in *P. fimata*. The most abundant were 16:0, 18:1ω9 and 18:2ω6,9, these three together represented 78 - 85% of total NLFAs. Prevalence of C16 and C18 FAs has been documented in lipids of all insect orders (Fast, 1970; Thompson et al., 1973; Stanley-Samuelson et al., 1988). Holmstrup et al. (2002) found 14 different FAs in *Folsomia candida*, with 91% of the total NLFAs being 16:0, 18:1ω9, 18:2ω6,9, 18:0, 20:4ω6,9,12,15 and 20:5ω3,6,9,12,15. These FAs represented 92% of the total NLFAs in our study. However, we did not detect the 20:5ω3,6,9,12,15. Dietary fatty acids can be absorbed and incorporated without modification into body tissues of insects (Stanley-Samuelson et al., 1988). Ichneumonid wasps were shown to match precisely the fatty acid profiles of their hosts, retaining no characteristic pattern of their own (Thompson, 1973). This suggests that the lipid components of Collembola may reflect the pattern of their food source. In our experiments the Collembola generally had a more diverse FA profile then their diet. They contained more long-chain polyenoic FAs, especially with 20 carbon chain length, which are typical for animals. Our data indicate that *P. fimata* is able to synthesise 16:1ω7, 18:3ω6,9,12, 20:0, 20:2ω6,9 and 20:4ω6,9,12,15 fatty acids, as these were not present in the fungal diet. Several insect species convert C18 polyunsaturated FAs to their C20 products, with elongation and desaturation pathways resembling those in mammals (Dadd, 1983; Stanley-Samuelson and Nelson, 1993). Linoleic acid, the major precursor in this metabolic pathway, and an essential fatty acid for many animals, was in ample supply in the fungal host tissue. Therefore, we do not know if the Collembola were capable of the methyl-desaturation of oleic acid or if they incorporated the dietary fatty acid. De novo synthesis of linoleic and linolenic acid via oleic acid has been reported in some insects (Blomquist et al., 1982; Stanley-Samuelson, 1988).

2.4.3 Influence of starvation on *P. fimata*

Generally, starvation leads to the use of food reserves, such as glycogen and lipids. In the first period of food depletion Collembola are known to use glycogen and afterwards neutral lipids of storage fats (Testerink, 1981). In our investigation the Collembola were starved for up to 28 days and the fresh weight, dry weight and fatty acid composition was analysed. Dry weight showed a strong decline, whereas fresh weight showed minor changes except of a decrease on day 8 to 44% of the initial weight. In part this may have been caused by the fact that fresh weight is not constant due to differences in metabolic status or age of individual Collembola (Hillingsøe and Holmstrup, 2003), and physiological variation during moulting (Verhoef, 1981). However, the data suggest a high loss in body water content after 8 days of starvation. Correspondingly, a distinct decrease in body size was observed, indicating that the water loss may be caused by elevated evaporation due to an increase in surface-to-volume ratio (Verhoef and Li, 1983; Hillingsøe and Holmstrup, 2003). After 14 days the fresh weight of the Collembola was almost as high as at the start of the experiment. As the dry weight declined continuously and the fresh weight remained more or less constant, the relative body water content of the Collembola increased during the starvation period. This has been observed for other Collembola, previously (Vannier and Verhoef, 1978; Verhoef and Li, 1983; Lavy et al., 1997; Hillingsøe and Holmstrup, 2003). Verhoef and Li (1983) suggested this to be a mode of blood volume regulation in *Orchesella cincta* (Collembola), which was in agreement with the constant blood osmolarity they observed.

Neutral lipids represent a large energy reserve, which is used when food is in short supply (Holmstrup et al., 2002). These NLFAs are located in fat bodies, which are associated with the midgut of Collembola (Hopkin, 1997). Energy can also be stored as glycogen, which is used in periods of short food deprivation. Testerink (1981) documented, that the lipid content of *O. cincta* stays constant during the first three days of starvation, then declines until day seven and afterwards remains constant. He also measured the glycogen content, which decreased within three days to zero. This is consistent with our results, where the dry weight of *P. fimata* declined rapidly during the first three days and the amount of neutral lipids stayed constant, indicating that during this period *P. fimata* used glycogen as energy source. The decrease in storage lipids and body weight due to food depreviation is generally strongest in the first week (Testerink, 1981; Lavy et al., 1997; Hillingsøe and Holmstrup, 2003). We observed a comparable pattern in our experiment, where storage lipids and individual dry weight declined until the second week, and then remained constant. Animals respond to food deprivation by slowing down physiological processes, such as metabolic rate (Verhoef and Li, 1983; Lavy et al., 1997), respiration rate (Hubert and Šustr, 2001) and moulting rate (Booth and Anderson, 1979; Verhoef et al., 1988). Our results suggest that *P. fimata* takes about two weeks to adapt its metabolism to food deprivation. In contrast to NLFAs, the amount of PLFAs was not affected by starvation, likely due to their function as structural components of cell membranes, which are not used for energy supply.

In general, the proportion of most NLFAs did not change during starvation suggest-

ing that fatty acids were metabolized indiscriminately. Similarly, Canavoso et al. (1998) reported a remarkable decline in lipid stores in Triatominae (Heteroptera), without noticeable changes in their composition. The PLFA pattern also remained very constant during starvation except for oleic acid, which may have been used as precursor for the long-chain polyunsaturated FAs that increased after one week of food depletion.

2.4.4 Influence of different nitrogen content of the food source

Many fungi adapt physiologically to a wider range of concentrations of nitrogen, which may lead to variations in the nutritional quality of hyphae and affect food preferences of fungal feeding Collembola (Shaw, 1988). The C/N ratio of hyphae is an important determinant of food quality, with a low ratio indicating high protein content. In *P. fimata* the content of NLFAs was significantly increased when fed with fungi with low N content. Lavy and Verhoef (1996) observed for the Collembola *O. cincta* that it compensates for low dietary nitrogen by consuming more food to maintain a relatively constant amount of protein and as a consequence accumulates more fat. This mechanism of nutritional compensation also occurs in other insects (Simpson and Abisgold, 1985; Karowe and Martin, 1989; Simpson and Simpson, 1990). Nutritional compensation likely was responsible for the increase in the amount of NLFAs in *P. fimata* fed with low nitrogen diet in our experiment. However, the amount of PLFAs increased with increasing nitrogen content of hyphae. Presumably, the N content of the food source also affected the physiological status of the Collembola. It has been documented to stimulate the growth and fecundity of fungivores (Booth and Anderson, 1979; Lavy and Verhoef, 1996). Food richer in nitrogen likely induce the production of more cells, i.e. membranes, which contain PLFAs as major compound. The activated metabolism also may need more mitochondria to provide energy resources for the cells. We observed a higher proportion of C20 eicosanoids and other unsaturated fatty acids, which was mirrored by a significant increase in the *UI* of PLFAs with enhanced N availability in the fungal food source. The *UI* is a measure for membrane fluidity, and changes indicate altered structural or functional demands in biomembranes, likely caused by the alterations in Collembola metabolism due to variations in food quality.

Not only the ratio of storage lipids (NLFAs) to membrane lipids (PLFAs) differed due to the N content of the food source, also the NLFA pattern of the Collembola was affected significantly. The *UI* decreased from 1.06 to 0.77 with decreasing C/N ratio, mainly because of the significant increase in 16:0 and the significant decrease in 18:1ω9 and 18:2ω6,9. In a different system, growing a flagellate under altered N regimes, Parrish et al. (1998) also observed an increase in saturated FAs with low food N content. However, the obvious changes in the NLFA profile of the Collembola cannot be explained by the changes in the lipid profile of the fungal food, as the pattern of major FAs in the fungus was vice versa. This suggests that the fatty acid pattern of *P. fimata* does not depend entirely on its food source. Similarly, the fatty acid profile of the culture medium was different from the tissue fatty acid pattern in the Tenebrionid beetle *Zophobas atracus*, indicating that the insect fatty acid pattern does not mirror exactly the fatty acid pattern

of the diet (Howard and Stanley-Samuelson, 1996). Firstly, this might be because the fatty acid composition is adjusted to meet the need of individual tissues. Secondly, Collembola consume less food when the nitrogen content of the food source is high (Lavy and Verhoef, 1996) and as a consequence they ingest less fatty acids. The fatty acids needed to build membranes are used directly and are not stored as neutral lipids and therefore are not detected in the NLFA fraction. Only the excess FAs are stored, which may diminish the signal of the fungal pattern.

2.5 Conclusions

Starvation did not affect the fatty acid composition of the storage lipids in Collembola, whereas the C/N ratio of the fungal food source altered the NLFA profile distinctly. However, the NLFA pattern of *P. fimata* was more affected by the N content than by the FA profile of the fungal resource, likely due to compensatory responses to variations in dietary nitrogen. This indicates that the same fungus grown on different substrates may induce different fatty acid patterns in the storage lipids of its fungal grazers. Generally, the PLFA pattern of the Collembola was only slightly altered by food quality or starvation. This implies that PLFAs, i.e. membrane lipids, are less affected by nutritional variations than NLFAs. However, PLFA patterns may change in the long-term due to altered metabolism caused by food deprivation. Our results suggest that the influence of dietary lipid composition on the pattern of consumers may be altered by their metabolism and physiological status diminishing the signal of individual fatty acids. We conclude that fatty acid patterns are not fixed and that diet and development exert strong influence on fatty acid profiles. More information on the regulation of these processes is needed to assign specific fatty acids as biomarkers in trophic cascades.

Chapter 3

Effects of food quality, starvation and life stage on stable isotope fractionation in Collembola

Abstract

Naturally-occurring stable isotopes of carbon and nitrogen are powerful tools to investigate food webs, where the ratio of $^{15}N/^{14}N$ is used to assign trophic levels and of $^{13}C/^{12}C$ to determine the food source. A shift in $\delta^{15}N$ value of 3.4‰ is generally suggested as mean difference between two trophic levels, whereas the carbon isotope composition of a consumer is assumed to reflect the signal of its diet.

This study investigates the effects of food quality, starvation and life stage on the stable isotope fractionation in fungal feeding Collembola. The fractionation of nitrogen was strongly affected by food quality, i.e. the C/N ratio of the fungal diet. Collembola showed enrichment in the heavier isotope with increasing N concentration of the food source. $\Delta^{15}N$ varied between 2.4‰, which assigns a shift in one trophic level, and 6.3‰, suggesting a shift in two trophic levels. Starvation up to 4 weeks resulted in an increase in the total $\delta^{15}N$ value from 2.8 to 4.0‰. Different life stages significantly affected the isotope discrimination by Collembola with juveniles showing a stronger enrichment ($\Delta^{15}N = 4.9‰$) compared to adults ($\Delta^{15}N = 3.5‰$). $\Delta^{13}C$ varied between -2.1 and -3.3‰ depending on the food quality, mainly due to compensational feeding on low quality diet. During starvation $\delta^{13}C$ value decreased by 1.1‰, whereas the life stage of Collembola had no significant effect on isotopic ratios.

The results indicate that the food resource and the physiological status of the consumer have important impact on stable isotope discrimination. They may cause differences in fractionation rate comparable to trophic level shifts, a fact to consider when analysing food web structure.

3.1 Introduction

Measurement of naturally occurring stable isotopes of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) are used as a tool to investigate dietary pattern and trophic relationships within ecosystems (Gannes et al., 1997, 1998; Ponsard and Arditi, 2000; McCutchan et al., 2003). Stable isotope ratios in animals change depending on their position in the food web and can thus provide trophic-level information of consumers (Wada et al., 1993; Post, 2002). Animals typically show fractionations ranging from 2‰ to 5‰ between dietary and tissue nitrogen (DeNiro and Epstein, 1981). Due to this nitrogen in the tissues of animals higher in the food chain tend to be enriched in the heavier isotope compared to animals at lower trophic levels (Minagawa and Wada, 1984). This discrimination in nitrogen stable isotopes is caused by a preferential removal of "light" amine groups by the enzymes responsible for amino acid deamination and transamination (Macko et al., 1986, 1987).

Generally, the carbon isotopic composition of the whole body in animals reflects the $\delta^{13}C$ value of their diet, as they differ more from diet to diet than due to trophic differences. The mean trophic shift for $\delta^{13}C$ is about +1‰ (DeNiro and Epstein, 1978; Post, 2002). However, the difference between the $\delta^{13}C$ values of biochemical fractions in an animal and its food source may be as large as -1.2 to +4.3 ‰ (Pearson et al., 2003). The trophic transfer of $\delta^{13}C$ was used to get insight into soil food web structure (Ponsard and Arditi, 2000; Scheu and Falca, 2000; Schmidt et al., 2004) thereby recent studies focus on the feeding strategies of Collembola (Chamberlain et al., 2004; Scheu and Folger, 2004; Ruess et al., 2005a). Diet strongly influences stable isotope composition, but there are other factors, which change fractionation rates. Several studies showed that starvation and food quality affect the ratio of nitrogen isotopes, but the results are not uniform. Adams and Sterner (2000) investigated *Daphnia magna* fed with *Scenedesmus acutus*, and found lower enrichment of ^{15}N with high N concentration of the food, but an increase with time under starvation. Similar results were reported for geese (Hobson et al., 1993). On the other hand some authors described an increase of $\delta^{15}N$ value with increasing N concentration in the diet (Rothe and Gleixner, 2000; McCutchan et al., 2003; Pearson et al., 2003; Ruess et al., 2004). Gannes et al. (1997) conclude, that a difference in nitrogen isotopic ratios of two organisms does not necessarily indicate different diets, different trophic levels or different conditions. For instance the main biochemical form of nitrogenous waste is an important factor for fractionation, too, as ammonotelic organisms show lower ^{15}N enrichment than ureotelic or uricotelic organisms (Vanderklift and Ponsard, 2003; Ruess et al., 2004). Additionally, stable isotopes from the diet are fractionated differently among animal tissues (Gannes et al., 1997, 1998; Pearson et al., 2003). When lipids are synthesised from dietary carbohydrate, the isotopic composition of the synthesised fatty acid is depleted in ^{13}C due to the fractionation during the oxidation of pyruvate to acetyl coenzyme A (DeNiro and Epstein, 1977; Gannes et al., 1998; McCutchan et al., 2003). Lipids can be depleted by up to 10‰ relative to other animal tissues (Tieszen and Boutton, 1988). This indicates that the body condition of an animal (i.e. the amount of storage fat) can affect the whole body $\delta^{13}C$ signature distinctly (Focken and Becker,

1998; Gearing, 1991). In sum, individual protein and lipid balance has a strong impact on nitrogen and carbon stable isotope composition in animals (Gaye-Siessegger et al., 2004a,b).

These various factors influencing stable isotope discrimination by animals involves some uncertainty in the use of this method for ecological research and calls for more defined laboratory experiments. In this study we investigate the effects of food depletion (i.e. starvation) and quality (i.e. C/N ratio of fungal diet) on $^{15}N/^{14}N$ and $^{13}C/^{12}C$ stable isotope fractionation in the Collembola *Protaphorura fimata*. Additionally, the influence of different life stages is assessed. Food quality likely will alter consumption rates and nutritional status in Collembola. Starvation strongly affects their metabolism by changing the ratio of anabolic to catabolic processes and similarly different life stages will alter the balance between metabolic pathways. Thus, starvation, food quality and life stage are expected to affect the fractionation of stable isotopes in Collembola, which may result in patterns not related to the consumed diet.

3.2 Materials and Methods

3.2.1 Fungi

Two common soil fungal species were used as food for Collembola in the experiments: the ascomycete *Chaetomium globosum* Kunze and the basidiomycete *Agrocybe gibberosa* (Fr.) Fay. *C. globosum* was cultivated on Potato dextrose agar (PDA, Merck). *A. gibberosa* was grown on Pachlewska agar adjusted to different N concentrations in order to manipulate the fungal C/N ratio. Pachlewska agar is a nutrient rich medium that contains 20 g glucose, 5 g maltose, 0.5 g $MgSO_4 \cdot 7\,H_2O$, 1 g KH_2PO_4, 50 μg thiamine HCl, 0.5 ml of 1% w/v Fe citrate, 0.5 ml of 2% w/v $ZnSO_4$ and 15 g agar per 1 l distilled water. As nitrogen source we added 0.5, 0.75, 1.5 and 3 g NH_4 tartrate per 1 l (equivalent to 75, 150, 300, 600 μg N/g agar). For the analysis of stable isotopes, fungi were grown on agar covered by a membrane filter (Millipore, 0.8 μm) at 15°C in darkness. To harvest fungal material, the filters were stripped off the agar and the fungi were removed aseptically with a scalpel from the filter.

3.2.2 Collembola

Collembola of the species *Protaphorura fimata* were taken from laboratory cultures fed with bakers yeast. Specimens were put into plastic vessels (diameter 7 cm, height 4.5 cm) containing a layer of plaster mixed with activated charcoal (2:1) at the bottom. The vessels were kept at 15°C in darkness and kept moist with distilled water. Each vessel contained 10 individuals of *P. fimata*. This low density was chosen to avoid cannibalism due to crowding. Eggs, pellets and exuvia were removed once a week. Collembola were fed with fungal diet ad libitum. Round pieces of fungal mats with a diameter of 10 mm were cut out of the agar cultures under sterile conditions, put into the vessels and renewed

once a week. Collembola were fed with the specific diet for 6 weeks. Chamberlain et al. (2004) found a half life for bulk carbon turnover rate of 3.5 days. The isotopic composition of the Collembola in our experiments should therefore be only due to composition of the fungal food source.

3.2.3 Experimental set-up

Three experiments were carried out to investigate $^{15}N/^{14}N$ and $^{13}C/^{12}C$ fractionation in the Collembolan *P. fimata*. The first experiment investigated changes due to variations in food quality represented by an altered C/N ratio of the agar used as culture media for the fungus *A. gibberosa*. *P. fimata* was fed with *A. gibberosa* at four different C/N levels. The second experiment analysed the effects of starvation. The Collembola were reared with *C. globosum*, then the fungus was removed and animals were kept without food supply for additional four weeks. Specimens were sampled at day 0 (non-starved), 2, 4, 8, 14, 21 and 28. The third experiment investigated variations in stable isotope ratios with life stages. Eggs of *P. fimata* were kept in plastic vessels until hatching. Collembola hatched within one week were joint into the same life stage group. Specimens were fed with *C. globosum* for 4, 8 and 12 weeks after hatching and then sampled. For each experiment three replicates per treatment or sample date were performed and harvested destructively. Collembola (30 individuals per replicate) were frozen at -15°C until analysis.

3.2.4 Stable isotope measurement

For analysis of stable isotope signatures Collembola and fungi were dried at 60°C for 2 days, weighed into tin capsules, and stored in a desiccator until measurement. Isotope ratios were determined by a coupled system of an elemental analyser (NA 1500, Carlo Erba, Milan) and a mass spectrometer (MAT 251, Finnigan, Bremen). The system is computer-controlled, allowing on-line measurement of ^{15}N and ^{13}C. Stable isotope abundance is expressed using the δ notation with

$$\delta X\,[‰] = (R_{sample} - R_{standard})/R_{standard} \times 1000$$

with "X" representing ^{15}N or ^{13}C, and R_{sample} and $R_{standard}$ representing the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratios of the sample and standard, respectively.

For ^{15}N, atmospheric nitrogen served as the primary standard and acetanilide (C_8H_9NO, Merck, Darmstadt) for internal calibration. For ^{13}C, Peedee belemnite marine limestone (PDB) was used as the standard (Lajtha and Michener 1994). The mean standard deviation of samples of 10 - 200 μg N, the range of the samples analysed, is 0.2‰ (Reineking et al. 1993). Fractionation of stable isotopes between food source (A) and consumer (B) is described in terms of the difference in delta (δ) values using the Δ notation, where $\Delta = \delta_B$ - δ_A. A positive Δ value indicates a relatively greater concentration of the heavier isotope in B.

3.2.5 Statistical analysis

Differences in total N concentration, C/N ratio, δ^{13}C and δ^{15}N of fungi and Collembola were analysed using ANOVA. If significant effects were suggested, differences between means were analysed by Tukey's honestly significant difference test (HSD). Statistical analyses were performed using SAS (SAS Institute Inc., Cary, USA). Correlation was carried out using Pearson's correlation coefficient. This statistical analysis was performed using JMP for Macintosh (SAS Institute Inc., Cary, USA).

3.3 Results

3.3.1 Agar media and fungal food source

The total N concentration of the PDA medium was 1.1% and the C/N ratio was 30. δ^{13}C and δ^{15}N values were -17.6‰ and -0.3‰, respectively (Table 3.2). Pachlewska agar had a total N concentration of 0.01, 0.018, 0.032 and 0.06% in the media with 75, 150, 300 and 600 μg N/g agar, respectively. The corresponding C/N ratios were 150, 93, 53 and 29.

The total N concentration of the fungus *C. globosum* was 5.0%. C/N ratio, δ^{13}C, and δ^{15}N values were 7.9, -16.1‰ and -1.1‰, respectively. The proportion of total N of *A. gibberosa* ranged from 1.3 to 5.7% and increased with higher N concentration of the agar ($F_{3,8}$=11.52, P=0.0028, Table 3.1). Concentrations of 75 and 150 μg N/g agar resulted in a significantly higher C/N ratio of *A. gibberosa* (16 and 19, respectively, $F_{3,8}$=36.70, P<0.0001) compared to 300 and 600 μg N/g agar, where the C/N ratio was 9. The δ^{15}N value of *A. gibberosa* ranged from 0.1 to 3.1‰ and tended to decline with higher amounts of N in the agar ($F_{3,8}$=2.89, P=0.1021). The δ^{13}C values ranged between -11.0 and -13.1‰ and increased with the N concentration of the agar ($F_{3,8}$=4.69, P=0.0357).

3.3.2 Collembola

Food quality

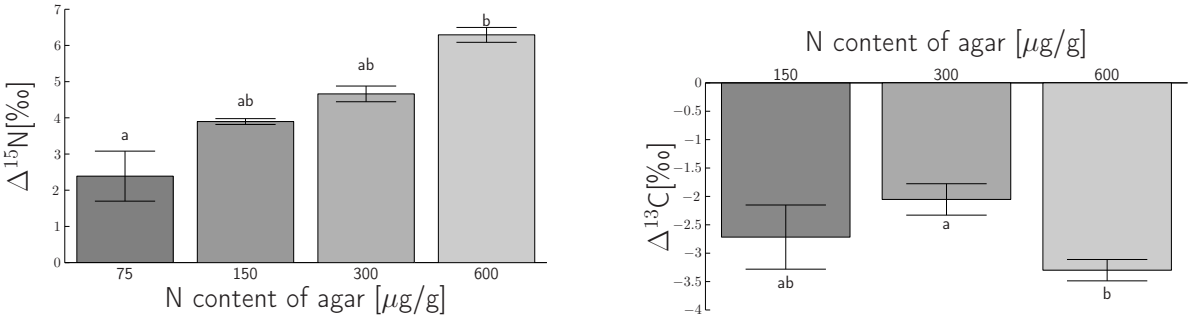
The N concentration of the fungal diet distinctly affected the N concentration of the Collembola (Table 3.1). Total N ranged between 6.0 and 8.2% and was high with increased amount of N in the fungal food source ($F_{3,8}$=26.01, P=0.0002). Correspondingly, the C/N ratio of the Collembola decreased from 9.2 to 4.1 with decreasing C/N ratio of the fungi ($F_{2,6}$=368.60, P<0.0001).

δ^{15}N in Collembola ranged between 5.5 and 7.3‰ and increased with higher N concentration in the diet ($F_{2,6}$=34.31, P=0.0005). The δ^{13}C values ranged between -13.2 and -14.7‰ and were lowest at intermediate C/N ratios ($F_{2,6}$=13.61, P=0.0059).

Collembola were enriched in ^{15}N and depleted in ^{13}C compared to their fungal food source (Fig. 3.1). There was a positive correlation between N concentration of the fungus and δ^{15}N value in Collembola ($F_{1,10}$=19.23, P=0.0014, R^2=0.66). Δ^{15}N increased from

	% N total	C/N-ratio	δ^{13}C	δ^{15}N
A. gibberosa				
75μg/g	1.3 ±0.9 c	16.2 ±2.7 a	-13.1 ±1.5 b	3.1 ±1.4 a
150μg/g	2.2 ±0.1 bc	19.0 ±0.4 a	-12.0 ±0.3 ab	2.2 ±0.4 a
300μg/g	5.7 ±1.8 a	9.0 ±0.5 b	-11.1 ±0.2 ab	2.6 ±2.2 a
600μg/g	4.3 ±0.4 ab	9.3 ±0.7 b	-11.0 ±0.2 a	0.1 ±0.5 a
P. fimata				
75μg/g	7.6 ±0.3 a	n.d.	n.d.	5.5 ±0.7 b
150μg/g	6.0 ±0.5 b	9.2 ±0.4 a	-14.7 ±0.6 b	6.1 ±0.1 b
300μg/g	8.2 ±0.3 a	5.0 ±0.2 b	-13.2 ±0.3 a	7.3 ±0.2 a
600μg/g	8.1 ±0.2 a	4.1 ±0.05 c	-14.3 ±0.2 b	6.4 ±0.2 ab

Table 3.1:
N content (% of dry weight), C/N ratio, δ^{13}C and δ^{15}N (± s.d.) of the fungus *Agrocybe gibberos*a grown on Pachlewska agar with different N content (75 μg/g, 150 μg/g, 300 μg/g; 600 μg N/g agar) and the Collembola *Protaphorura fimat*a fed with these fungi. Means within columns with the same letters are not significantly different from each other (Tukey's HSD, P<0.05)



(a) (b)

Figure 3.1:
Fractionation of ^{15}N and ^{13}C by the Collembola *Protaphorura fimata* compared to its fungal food source *Agrocybe gibberosa* grown on Pachlewska agar with different N concentration (75 μg/g, 150 μg/g, 300 μg/g, 600 μgN/g agar). Bars (± s.d.) with the same letters are not significantly different from each other. (Tukey's HSD, P<0.05)

	% N total	C/N-ratio	δ^{13}C	δ^{15}N
PDA	1.1 ±0.4	29.9 ±0.7	-17.6 ±0.1	-0.3 ±0.6
C. globosum	5.0 ±0.3	7.9 ±0.5	-16.1 ±0.2	-1.1 ±0.2
P. fimata				
Day 0	8.9 ±0.3 b	6.1 ±0.1 a	-18.0 ±0.05 a	2.8 ±0.4 a
Day 2	11.4 ±1.1 a	5.5 ±0.4 abc	-17.9 ±0.1 a	3.5 ±0.6 a
Day 4	11.1 ±0.8 ab	5.7 ±0.2 ab	-18.2 ±0.2 ab	3.4 ±0.5 a
Day 8	9.5 ±0.1 ab	5.4 ±0.1 abc	-18.7 ±0.3 ab	3.5 ±0.1 a
Day 14	11.1 ±1.4 ab	4.8 ±0.2 bc	-18.7 ±0.3 ab	3.8 ±0.4 a
Day 21	11.6 ±0.5 a	4.6 ±0.2 c	-18.7 ±0.1 ab	3.8 ±0.2 a
Day 28	10.1 ±0.6 ab	4.8 ±0.5 bc	-18.8 ±0.4 b	4.0 ±0.1 a

Table 3.2:
N content (in % of dry weight), C/N ratio, δ^{13}C and δ^{15}N (± s.d.) of agar medium (potato dextrose agar), fungus grown on that agar (*Chaetomium globosu*m) and the collembolan *Protaphorura fimata* fed with that fungus and starved then for up to 28 days. Means within colums with the same letters are not significantly different from each other (Tukey's HSD, $P<0.05$)

2.4 to 6.3‰ at higher concentrations of N in agar ($F_{3,8}$=55.26, $P<0.0001$). δ^{13}C values of *P. fimata* were lower than those of the fungal food source by -2.7, -2.1 and -3.3‰ in the treatments with 150, 300 and 600 μgN/g agar, respectively. The isotopic fractionation was significantly affected by the N concentration of the agar used as growth media for the fungus ($F_{2,6}$=8.12, P=0.0197).

Starvation

Starvation of *P. fimata* for a period of four weeks affected the isotopic composition of the animals and their total N concentration distinctly. The total amount of N increased from 8.9% at day 0 to 11.6% at day 21 ($F_{6,14}$=5.08, P=0.0058, Table 3.2). The C/N ratio ranged between 6.1 and 4.6 and decreased significantly with time during starvation ($F_{6,14}$=7.76, P=0.0008). The δ^{13}C value ranged between -17.9 and -18.8‰, with a slight decline in the heavier isotope ($F_{6,14}$=5.17, P=0.0054). The δ^{15}N values tended to increase during starvation from 2.8 to 4.0‰, particularly in the first week without food supply ($F_{6,14}$=2.13, P=0.1148). Non-starved Collembola (day 0) were depleted in ^{13}C by -1.9‰ and enriched in ^{15}N by 3.9‰ compared to the fungi used as diet.

Life stage

Total N concentration ranged between 10.1 and 10.4% and the C/N ratio between 4.9 and 5.2 and both were not significantly affected by age of the Collembola. The δ^{15}N values were 3.8, 4.3 and 2.4‰ and δ^{13}C values were -17.4, -17.6, and -17.1‰ for 4, 8 and 12

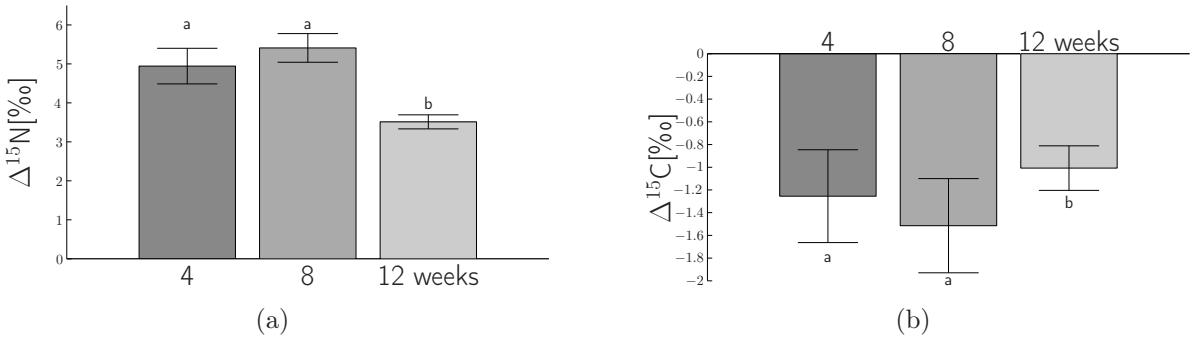


Figure 3.2:
Fractionation of ^{15}N and ^{13}C of the Collembola *Protaphorura fimata* in different life stages (4, 8, 12 weeks old) compared to its fungal food source *Chaetomium globosum*. Bars (± s.d.) with the same letters are not significantly different from each other.(Tukey's HSD, $P<0.05$)

weeks old individuals, respectively. The enrichment in ^{15}N compared to the fungal diet was 4.9, 5.4 and 3.5‰ in 4, 8, 12 week old Collembola with a significant decrease at week 12 ($F_{2,6}$=23.35, P=0.0015, Fig. 3.2). Collembola were depleted in ^{13}C compared to the fungal diet between -1.5 to -1.0‰.

3.4 Discussion

3.4.1 Food quality

Fungi adapt physiologically to a wide range of concentrations of nitrogen, which in turn results in different C/N ratios in fungal hyphae. The latter is an important determinant of the food quality for fungal feeding microarthropods, with a low ratio indicating high protein content. Several studies have demonstrated the importance of fungi in Collembola nutrition (Visser and Whittaker, 1987; Chen et al., 1995; Klironomos and Kendrick, 1995) and food selection was shown to be influenced by the nutritional status of the fungal hyphae (Booth and Anderson, 1979). These variations in the nutritional quality of hyphae can affect food preferences of fungal feeding Collembola (Shaw, 1988). In our experiment a high N concentration of the fungal diet increased the N concentration and the δ^{15}N value in Collembola. This is in line with other studies which reported an enrichment in ^{15}N in animals fed with high food quality or high N concentration (McCutchan et al., 2003, Pearson et al., 2003, Rothe and Gleixner, 2000), however, contrasting effects were also observed (Adams and Sterner, 2000; Fantle et al., 1999; Hobson and Clark, 1992; Webb et al., 1998). These contradictory findings likely resulted from the wide range of food quality tested. First, fractionation rate in animals is higher when feeding on low quality food since it resembles starvation, where body tissue including proteins is metabolised (Webb et al., 1998). Second, if low but sufficient amounts of proteins are incorporated the amino acids are used entirely for tissue formation, and animals therefore do not fractionate ^{15}N (Sponheimer et al., 2003; Ostrom et al., 1997). Third, if high amounts of proteins are incorporated, animals fractionate the protein N in excess

when storing the carbon and energy gained from amino acids as fat, resulting in ^{15}N enrichment. Gaye-Siessegger et al. (2003, 2004a) found a decrease in $\delta^{15}N$ value with increasing feeding rate in fish. In our experiments Collembola were fed at libidum and the amount of N in the food source was successively enlarged. Individuals on low protein diet likely reserved dietary protein for tissue maintenance rather than catabolising it and excreting the nitrogen therein ("protein sparing"; Gannes et al., 1997), which results in the observed low fractionation in ^{15}N. On the other hand Collembola feeding on fungal hyphae with high food quality presumably catabolized excess dietary protein and by this had a higher fractionation rate. Similar to the present study Ruess et al. (2004) reported of a positive correlation between the N concentration in the diet and the $\delta^{15}N$ value in nematophagous and fungivorous Collembola. Overall, the results suggest that the protein balance of a consumer significantly affects the trophic level shift of ^{15}N, which is in line with Pearson et al. (2003) and Gaye-Siessegger et al. (2004b).

In contrast to $\delta^{15}N$, *P. fimata* had a stronger depletion in $\delta^{13}C$ on low and high quality diet compared to diet of intermediate quality. The decrease in ^{13}C with lower food quality likely resulted from an accumulation of storage fat (Schmidt, 1999; Kelly, 2000). From the present experiment NLFAs and PLFAs were investigated (Haubert et al., 2004, see Chapter 2), which also supports the accumulation of storage fat as indicated by a distinct increase in the amount of neutral lipids. Fatty acids are known to be depleted in ^{13}C (DeNiro and Epstein, 1977), which affects the $\delta^{13}C$ value of the bulk biomass (Rau et al., 1991; Focken and Becker, 1998). *O. cincta* (Collembola) compensates for low dietary nitrogen by consuming more food to maintain a relatively constant amount of protein in the diet and as a consequence accumulates more fat (Lavy and Verhoef, 1996). Nutritional compensation also occurs in other insects (Simpson and Abisgold, 1985; Karowe and Martin, 1989; Simpson and Simpson, 1990). Bååth et al. (2003) proposed the use of fatty acids to indicate physiological conditions (e.g. nutrient status) in soil fungi by the ratio of neutral lipids (NLFA) to phospholipids (PLFA). We found an increase in the NLFA/PLFA ratio from 2.4 to 173 with decreasing food quality (Haubert et al., 2004, see Chapter 2), which suggests compensatory feeding by Collembola.

Surprisingly, the strongest decline in ^{13}C in *P. fimata* was with high quality diet, i.e. the lowest amount of (^{13}C depleted) body fat stored in tissues. Possibly, this was due to an altered metabolism. Presumably, the N concentration of the food source affected the physiological status of the Collembola and stimulated growth and fecundity (Booth and Anderson, 1979; Lavy and Verhoef, 1996). In fact, rearing *P. fimata* with high quality diet altered phospholipid fatty acid patterns (Haubert et al., 2004, see Chapter 2). Food richer in nitrogen likely induces the production of more cells, i.e. membranes, which contain PLFAs as major compound.

3.4.2 Starvation

When *P. fimata* was starved for 28 days the total N concentration increased and the C/N ratio declined, which was due to a reduction in total carbon content of the animals. Obviously, Collembola used body glycogen and lipids as energy resource, resulting in a decrease in the amount of neutral lipids, i.e. deposit fat (Haubert et al., 2004, see Chapter 2). Starvation distinctly changes the ratio between catabolism and anabolism in animals. Molecules with light isotopes are preferentially catabolized, while molecules with heavier isotopes remain in the body (Webb et al., 1998). δ^{15}N value increased during starvation, which is in line with findings for spiders (Oelbermann and Scheu, 2002), polychaetes (Olive et al., 2003), and birds and mammals (Hobson and Clark, 1992; Ambrose, 1993; Hobson et al., 1993; Cormie and Schwacz, 1996). It is well documented that starvation increases the enrichment in ^{15}N, as amino acids derived from protein breakdown are deaminated (Hobson et al., 1993).

The δ^{13}C value of bulk animals in our experiment decreased with starvation by 0.8‰, particularly in the first week, when Collembola used their storage fat (Haubert et al., 2004, see chapter 2). As lipids are generally depleted in the heavier isotope compared to bulk animals, an increase in ^{13}C as found in spiders (Oelbermann and Scheu, 2002), polychaetes (Olive et al., 2003) and fish (Gaye-Siesegger et al., 2004a) is to be expected, but did not occur our experiment.

3.4.3 Life stage

P. fimata fractionated ^{15}N of the fungal food source differently depending on their life stage. Compared to their diet 12-week-old Collembola fractionated ^{15}N less (1.3‰) than the 8 and 4-week-old individuals (3.2 and 2.7‰, respectively). This contradicts findings of Ponsard and Averbuch (1999) who postulated that growing animals have the same δ^{15}N values as those of adults when fed the same diet. In line with this statement Minagawa and Wada (1984) found no changes in the δ^{15}N value of marine mussels depending on age. Contrasting to this and to our results Scheu and Folger (2004) observed the fractionation in adult Collembola to exceed that of juveniles by 2.28 ‰, and Oelbermann and Scheu (2002) reported an increase in ^{15}N in spiderlings with age. They suggest that variations in ^{15}N within life stage may be caused by differences in food source. However, in our experiment the Collembola were maintained on the same diet. Likely, the observed changes in ^{15}N fractionation were due to a reduced metabolism in older Collembola. The 12-week-old individuals stopped to lay eggs and moulting was infrequent. Fractionation in ^{13}C did not change significantly with age. Again this contradicts findings from Scheu and Folger (2004) where fractionation was generally more pronounced in adult *Heteromurus nitidus* (Collembola) (-2.7‰) than in juveniles (-0.91‰).

3.5 Conclusions

It is generally accepted that the difference between trophic levels in δ^{15}N is about 3.4‰. However, our experiments showed that differences in animal physiology might result in a fractionation comparable to trophic level shifts. The extrapolation of the trophic level by δ^{15}N or the reconstruction of the diet by δ^{13}C without the knowledge of the physiological conditions of an animal may lead to erroneous results. However, the implication of our laboratory findings to the field remains to be explored. We investigated few feeding links and the postulated mean trophic level fractionation for carbon and nitrogen (1‰ and 3‰, respectively) may still be a valid approximation when applied on entire food webs with multitrophic pathways and many species (Post, 2002). We suggest that to provide the high level of resolution required, stable isotopes must be used in combination with other information, such as gut content analysis or morphological structures of mouthparts, but in particular with fatty acid composition of the consumer and its diet (Ruess et al., 2002).

Chapter 4

Trophic shift of stable isotopes and fatty acids in Collembola on bacterial diets

Abstract

Soil food webs are difficult to observe due to the cryptic habitat and the small size of the animals. Fatty acid (FA) analysis is a promising tool to study trophic relationships in soil food webs. We determined FA biomarkers to trace bacterial food sources (*Bacillus megaterium*, *Pseudomonas putida*, *Enterobacter aerogenes*) of Collembola (*Heteromurus nitidus*, *Protaphorura fimata*, *Folsomia candida*). In addition, δ^{15}N, δ^{13}C, C/N ratio, body weight and NLFA/PLFA ratio (neutral lipid/phospholipid fatty acids) of Collembola were assessed. These measures indicated that *P. putida* ranked first, *B. megaterium* second and *E. aerogenes* third in food quality. Fatty acids specific for bacteria were found in the neutral lipid fatty acids of the Collembola reflecting the respective bacterial diet. Biomarker FAs for gram-positive bacteria were methyl branched i14:0, i15:0, a15:0 and i17:0. Consumption of gram-negative bacteria was reflected by the cyclic form cy17:0 (*E. aerogenes*, *P. putida*) and by 16:1ω5 (*P. putida*).

4.1 Introduction

Trophic relationships in soil food webs are difficult to assess. Different approaches have been used to solve this problem, most comprehensively stable isotope analysis. Naturally occurring stable isotopes of nitrogen (^{15}N/^{14}N) and carbon (^{13}C/^{12}C) provide insight into dietary and trophic relationships within food webs (Gannes et al., 1997, 1998; Ponsard and Arditi, 2000; McCutchan et al., 2003). Animals higher in the food chain tend to be enriched in the heavier isotope compared to animals at lower trophic levels (Gannes et al., 1998; Minagawa and Wada, 1984). Generally, the fractionation between resource and consumer tissue nitrogen is in the range of 2 - 5‰ and indicates the trophic level of an

animal (DeNiro and Epstein, 1981; Post, 2002). In contrast, the isotopic composition of carbon of the whole body in small animals closely resembles that of their diet; with an overall enrichment of about +1‰ (DeNiro and Epstein, 1978; Post, 2002). The trophic transfer of δ^{15}N was used to get insight into the structure of soil food webs (Ponsard and Arditi, 2000; Scheu and Falca, 2000; Schmidt et al., 2004). Recent studies focused on the feeding strategies of Collembola (Chamberlain et al., 2004; Scheu and Folger, 2004; Ruess et al., 2005a). Diet strongly influences stable isotope composition, but there are other factors, which also effect fractionation rates. A number of studies showed that starvation and food quality affect the $^{15}N/^{14}N$ ratio in arthropods (Haubert et al., 2005, see Chapter 3; Adams and Sterner, 2000; Ruess et al., 2004a; Rothe and Gleixner, 2000), birds (Hobson et al., 1993, Pearson et al., 2003) and fish (Gaye-Siesegger et al., 2003, 2004 a, b).

Only recently, FA analysis has been suggested as a promising tool to investigate trophic interactions in soil. Energetically it is more efficient to incorporate dietary FAs without modification into body tissue, which leads to a lipid pattern in the consumer that reflects the composition of its diet (Stott et al., 1997). The specificity of FA synthesis and composition in different taxonomic groups is the basis for their wide use as biochemical marker of trophic and metabolic interactions in aquatic food webs (Desvilettes et al., 1997, Leveille et al., 1997). FA markers were applied to map the transfer of carbon through aquatic food webs and to understand the feeding strategies of consumers (Ederinton et al., 1995; Gladyshev et al., 1999, 2000). Trophic transfer of FAs from the fungal food source to nematode or Collembola grazers was reported by Ruess et al. (2002, 2004, 2005a) and Chamberlain et al. (2004), and FA biomarkers for different feeding strategies have been assigned (Chamberlain et al., 2005, Ruess et al., 2005b).

Here we investigate the trophic transfer of stable isotopes and fatty acids from bacteria to Collembola. They appear to feed on a wide spectrum of food resources, including Protozoa, Nematoda, Rotifera, Enchytraeidae, bacteria, fungi, algae, plant litter and living plant tissue (Rusek, 1998; Chahartaghi et al., 2005). Fungi generally are regarded as the most important diet of Collembola (Chen et al., 1995; Klironomos and Kendrick, 1995). In this study we offered common soil bacteria as food source to Collembola and assessed the suitability of the bacterial diet by analysing the NLFA/PLFA ratio as a measure of the metabolic status and the fractionation in ^{13}C and ^{15}N as an indicator of food quality. FA profiles of diet and grazer were compared in order to identify FA biomarkers for bacterial feeding in Collembola.

4.2 Material and Methods

4.2.1 Bacteria

Three different widespread soil bacteria were used as food for Collembola in the experiments: the gram-positive bacterium *Bacillus megaterium* (de Bary, 1884) and the gram-negative bacteria *Enterobacter aerogenes* (Hormaeche and Edwards, 1960) and *Pseu-*

domonas putida (Trevisan, 1889; Migula, 1895). Bacteria were cultivated in Standard I nutrient broth (Merck, Darmstadt, Germany) at room temperature for 3 days. They were harvested from the liquid cultures by centrifugation (3000 rpm, 3 min), and the pellet washed twice with sterile distilled water to remove traces of nutrient solution.

4.2.2 Collembola

The Collembola species *Protaphorura fimata* (Gisin, 1952), *Heteromurus nitidus* (Templeton, 1835) and *Folsomia candida* (Willem, 1902) were taken from laboratory cultures fed with bakers yeast. Specimens were put into plastic vessels (diameter 7 cm, height 4.5 cm) containing a layer of plaster mixed with activated charcoal (2:1) at the bottom. Vessels were stored at 15°C in darkness and kept moist with distilled water. Each vessel contained 20 individuals of Collembola. Eggs, pellets and exuvia were removed with a brush once a week. Collembola were fed with bacterial diet ad libitum for 6 weeks. Experimental design was as follows: 4 replicates × 3 bacteria species × 3 Collembola species, with a final destructive sampling. Bacterial suspension (100 μl) was pipetteted on a piece of cellulose filter paper and put into the plastic vessels. The diet was renewed three times a week. After harvest the Collembola were frozen at -20°C until analysis.

4.2.3 Analysis of fatty acid patterns

Cellular lipids of Collembola were extracted by shaking in 5 ml single phase extraction solvent (chloroform/methanol/0.05 M phosphate buffer (pH 7.4) 1:2:0.8) overnight. The solvent was then transferred to new tubes and samples were re-extracted by shaking for 2-3 h with additional 2.5 ml. Extraction solvents of both steps were combined, 0.8 ml distilled water and 0.8 ml $CHCl_3$ were added, and samples centrifuged at 1500 rpm for 5 min. Samples were allowed to stand and separate. Then the top two phases were removed and the chloroform fraction of each sample was transferred to a silica acid column (0.5 g silicic acid, mesh size 100-200 μm). Lipids were eluted with 5 ml chloroform (neutral lipids), 8 ml acetone (glycolipids) and 5 ml methanol (phospholipids). The chloroform and methanol fraction was evaporated (50°C, vacuum 200 hPa) in a Labconco RapidVap (Labconco, Kansas City, USA). The NLFA and PLFA fractions of the Collembola and the whole cellular lipids of the bacteria were saponified and methylated following the procedures given for the Sherlock Microbial Identification System (MIDI Inc., Newark, USA). Saponification of lipids was conducted in a sodium hydroxide-methanol solution (45 g sodium hydroxide, 150 ml methanol, 150 ml distilled water) at 100°C for 30 min, followed by acid methanolysis in HCl-methanol (325 ml 6.0 N hydrochloric acid, 275 ml methyl alcohol) at 80°C for 10 min. The fatty acid methylesters were extracted into hexane-methyl tertiary butyl ether (1:1) and washed with aqueous NaOH (10.8 g sodium hydroxide, 900 ml distilled water). The lipid-containing phase was then transferred to test tubes and stored at -20°C until analysis.

Fatty acid methyl esters were analysed by gas chromatography (GC) using the Sherlock Microbial Identification System consisting of a Hewlett Packard 5890 Series II gas

chromatograph and flame ionisation detector equipped with an HP Ultra 2 phenyl methyl silicone fused capillary column (25 m × 0.2 mm i.d., film thickness 0.33 μm), an automated sampler and computer with associated software (Sherlock Pattern Recognition Software, MIDI). The fatty acid methyl esters were identified on the basis of their retention times and quantified. To verify correct identification of fatty acid methyl esters (chain length and saturation) a range of samples containing NLFAs and all samples with PLFAs were analysed by GC-mass spectrometry using an Agilent Series 6890 GC System and 5973 Mass Selective Detector, equipped with a HP5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). A mass range of 40 to 400 m/z was monitored in scan mode.

4.2.4 Stable isotope measurement

For analysis of stable isotope signatures Collembola and bacteria were dried at 60°C for 2 days in tin capsules, weighed and stored in a desiccator until measurement. Isotope ratios were determined by a coupled system of an elemental analyser (NA 1500, Carlo Erba, Milan) and a mass spectrometer (MAT 251, Finnigan, Bremen). The system is computer-controlled, allowing on-line measurement of ^{15}N and ^{13}C. Stable isotope abundance is expressed using the δ notation with

$$\delta X\,[‰] = (R_{sample} - R_{standard})/R_{standard} \times 1000$$

with "X" representing ^{15}N or ^{13}C, and R_{sample} and $R_{standard}$ representing the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratios of the sample and standard, respectively.

For ^{15}N, atmospheric nitrogen served as the primary standard and acetanilide (C_8H_9NO, Merck, Darmstadt) for internal calibration. For ^{13}C, Peedee belemnite marine limestone (PDB) was used as the standard (Lajtha and Michener 1994). The mean standard deviation of samples of 10 - 200 μg N, the range of the samples analysed, is 0.2‰ (Reineking et al. 1993). Fractionation of stable isotopes between food source (A) and consumer (B) is described in terms of the difference in delta (δ) values using the Δ notation, where $\Delta = \delta_B$ - δ_A. A positive Δ value indicates a relatively greater concentration of the heavier isotope in B.

4.2.5 Statistcal analysis

Data were analysed using ANOVA and successive Tukey's honestly significant difference test with SAS (SAS Institute Inc., Cary, USA). Discriminant function analysis (DFA) was carried out with STATISTICA 7.1 (StatSoft Inc., Tusla, USA). Fatty acids were used as variables for discrimination and the different bacterial food sources as groups. Multivariate fitting was performed by MANOVA, significances are given according to Wilk's test.

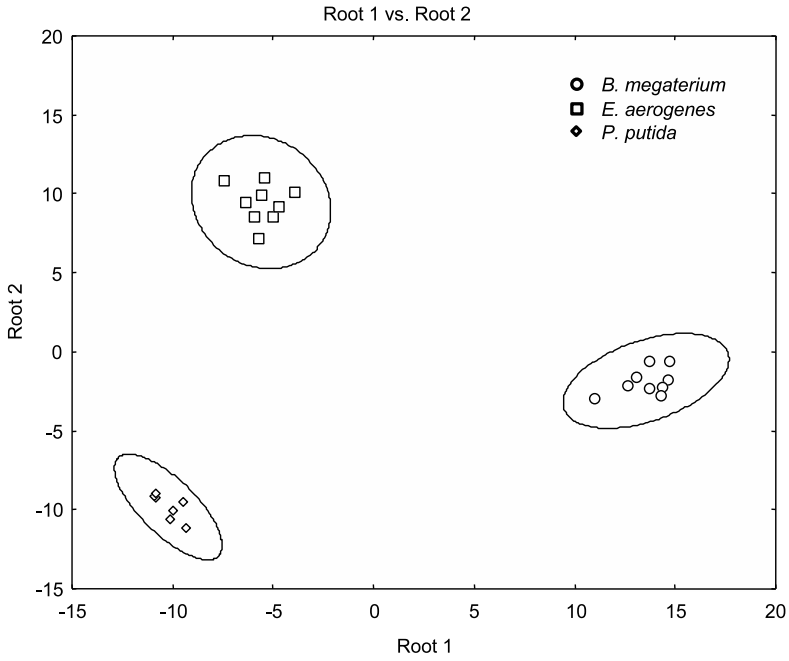


Figure 4.1:
Discriminant Function Analysis (DFA) of bacterial food sources *Bacillus megaterium*, *Enterobacter aerogenes* or *Pseudomonas putida* (groups, n = 3) based on the neutral lipid fatty acid composition (variables, n = 14) of Collembola (model: $F_{28,18}$=58,7 P<0.0001, data see Table 4.2) FAs with an abundance below 2.5% were not included in the analysis. Scatterplot shows ellipses with a = 0.05.

4.3 Results

4.3.1 Stable isotopes

Collembola fed and reproduced on all offered bacterial diets. Total dry weight of Collembola maintained with *E. aerogenes* (16 - 29 μg; Table 4.1) was significantly lower ($F_{8,16}$=14.5, P<0.0001) than in Collembola fed with *B. megaterium* (45 - 70 μg) and *P. putida* (36 - 54 μg). The C/N ratios of the bacterial food source were 3.6 (±0.02) for *B. megaterium*, 3.8 (±0.06) for *E. aerogenes* and 4.4 (±0.09) for *P. putida*. The isotopic composition and the C/N ratio of Collembola were influenced by the bacteria used as diet (Table 4.2). Collembola were depleted in ^{13}C when fed with *B. megaterium* (-0.5 to -1.0‰) or *P. putida* (0.1 to -0.4‰), but slightly depleted or enriched when fed with *E. aerogenes* (-0.2 to 1.1‰) ($F_{2,15}$=81.3, P<0.0001; Table 4.1). The fractionation of nitrogen isotopes was influenced significantly by the food source ($F_{2,18}$=119, P<0.0001) and was highest in Collembola fed with *E. aerogenes* (2.3-5.7‰), intermediate with *B. megaterium* (2.5-3.7‰) and lowest with *P. putida* (-0.4-0.7‰, Table 4.1).

4.3.2 Fatty acid composition

Bacteria contained FAs with a chain length between 12 and 19 carbon atoms (data not presented). The profile of the gram-positive *B. megaterium* comprised 17, and the gram-

	B. megaterium				*E. aerogenes*				*P. putida*				ANOVA		
	F. candida	*H. nitidus*	*P. fimata*		*F. candida*	*H. nitidus*	*P. fimata*		*F. candida*	*H. nitidus*	*P. fimata*		C	B	CxB
dry weight [μg]	50	70	45	a	16	29	19	b	47	54	36	a	**	***	
NLFA/PLFA ratio	13	26	33		23	6	5		13	34	20				
C/N ratio	6.6	-	5.7	a	2.3	3.2	3.4	b	5.4	4.7	5.7	c	**	***	
Δ^{13}C	-1.0	-	-0.5	a	-0.2	0.6	1.1	b	-0.4	-0.4	0.1	b	***	***	*
Δ^{15}N	3.7	3.1	2.5	b	5.7	2.3	5.2	a	0.7	-0.4	0.2	c	***	***	**

Table 4.1:

Dry weight, ratio of neutral lipid fatty acids (NLFA) to phospholipid fatty acids (PLFA) and C/N ratio of the Collembola *Folsomia candida*, *Heteromurus nitidus* and *Protaphorura fimata* fed with three different bacteria *Bacillus megatherium*, *Enterobacter aerogenes* or *Pseudomonas putida* and fractionation of ^{15}N and ^{13}C by the Collembola compared to its bacterial food source. Means within rows sharing the same letter showed no significant different effect of the bacterial food source (Tukey's HSD, $P<0.05$). ANOVA indicating significant effects of Collembola (C), bacteria (B) and interaction of bacteria and Collembola (CxB), with *, **, *** with $P<0.01$, 0.001, 0.0001.

negative *E. aerogenes* and *P. putida* 11 and 12 different FAs, respectively. The major FAs in *B. megaterium* were i13:0 (28%), i14:0 (12%), i15:0 (13%) and 16:0 (12%). Dominant FAs in the gram-negative *E. aerogenes* and *P. putida* were 16:1ω7 (18 and 11%, respectively), 16:0 (34 and 29%, respectively) and cy17:0 (17 and 31%, respectively).

Generally, Collembola contained more long-chain FAs with a higher degree of unsaturation than their bacterial diet. The neutral lipid profile consisted of 24 to 26 different FAs with 14 to 20 carbon chain length (Table 4.1). Major fatty acids were 16:0 (17-26%), 18:1ω9 (21-32%) and 18:0 (5-15%). The PLFA pattern of Collembola contained between 7 and 15 different FAs with a chain length between 14 and 20 carbon atoms (data not presented). Major FAs were also 16:0 (15-27%), 18:1ω9 (10-26%) and 18:0 (16-50%). The ratio of NLFA to PLFA is used as a marker for physiological conditions. *F. candida* showed no difference in NLFA/PLFA ratio with food source, whereas *H. nitidus* and *P. fimata* had a lower ratio when reared on *E. aerogenes* (5 and 6, respectively) compared with *P. putida* and *B. megaterium* (20 - 34).

4.3.3 Biomarker fatty acids

The fatty acid composition of the bacterial food source strongly affected the NLFA composition of the consumer. MANOVA revealed effects of Collembola ($F_{2,30}$=57.2, P=0.017), bacteria ($F_{2,30}$=921, P=0.001) and interactions ($F_{6,60}$=43.4, P<0.0001). In contrast, PLFAs were only little affected by diet (data not presented). Discriminant function analysis (DFA) of the NLFA patterns, with bacteria as groups in the model, distinctly separated the three food sources from each other (whole model: $F_{28,18}$=58.7, $P<$ 0.0001; *B. megaterium* × *E. aerogenes*: $F_{14,9}$=65.1, $P<$ 0.0001, *B. megaterium* × *P. putida*: $F_{14,9}$=72.3, P<0.0001; *E. aerogenes* × *P. putida*: $F_{14,9}$=45.3, $P<$ 0.0001; Fig. 4.1). The first two axes were significant with an eigenvalue of 121 and 68.8 for root 1 and 2, respectively ($P<$ 0.0001).

The gram-positive *B. megaterium* contained a high amount of methyl-branched (iso, anteiso) FAs, i14:0 (12%), a15:0 (5%), i15:0 (13%) and i17:0 (5%). These also occurred in the NLFAs of the Collembola (Table 4.2). The proportion of these FAs was significantly higher in Collembola fed *B. megaterium* than in those fed on *P. putida* and *E. aerogenes*. ($F_{8,18}$=112.82, P<0.0001; $F_{8,18}$=103.27, P<0.0001; $F_{8,18}$=266.45, P<0.0001 and $F_{8,18}$=327.61, P<0.0001, for i14:0, a15:0, i15:0 and i17:0, respectively).

The lipids of gram-negative bacteria comprised cyclic FAs, predominantly cy17:0 and cy19:0. These were also present in the NLFA of the consumer. In *E. aerogenes* 17% of the FAs were cyc17:0. This FA was present in each of the three Collembola species feeding on this bacterium (3.2-6.3%). *P. putida* contained a higher amount of cy17:0 (31%) than *E. aerogenes* leading to a significantly higher concentration in the Collembola of 12-14% (F=1099.98, P<0.0001). *E. aerogenes* also contained 6% of 16:1ω5, which was present in each of the Collembola species feeding on this food source (0.6-1.6%)

Bacterial FAs were also detected in small amounts in the PLFAs of Collembola (data not presented). Fed with *B. megaterium* Collembola comprised between 0.2 and 1% of

Food source				*B. megaterium*			*E. aerogenes*			*P. putida*		
Organism	*B. megaterium*	*E. aerogenes*	*P. putida*	*F. candida*	*H. nitidus*	*P. fimata*	*F. candida*	*H. nitidus*	*P. fimata*	*F. candida*	*H. nitidus*	*P. fimata*
12:0	1.3±0.0	3.3±0.3	7.4±0.2	-	-	-	-	-	-	-	-	-
i12:0	3.7±1.2	-	-	-	-	-	-	-	-	-	-	-
i13:0	27.9±3.7	-	1.8±1.8	-	0.4±0.1	0.6±0.1	-	-	-	-	-	-
a13:0	4.8±0.5	-	-	-	-	-	-	-	-	-	-	-
14:0	2.3±0.5	7.6±0.4	4.6±1.8	1.6±0.3	2.9±0.3	0.5±0.2	2.4±0.2	1.3±0.3	1.3±0.2	1.2±0.1	1.7±0.0	0.6±0.1
i14:0	**11.5±0.1**	**-**	**0.5±0.3**	**0.9±0.2**	**1.3±0.2**	**1.7±0.1**	-	-	**-**	**-**	**0.4±0.1**	**-**
15:0	-	2.3±0.1	1.6±0.1	0.5±0.1	-	-	1.0±0.3	1.9±0.4	1.3±0.2	-	0.5±0.0	-
i15:0	**12.5±0.6**	**-**	**0.9±0.2**	**6.5±0.2**	**5.2±0.3**	**5.7±0.3**	**1.3±0.5**	**0.2±0.4**	**1.7±0.3**	**3.1±0.7**	**1.3±0.3**	**1.4±0.6**
a15:0	**4.8±0.4**	**-**	**-**	**1.9±0.4**	**2.4±0.4**	**2.7±0.1**	**0.2±0.3**	-	**1.9±0.2**	**0.4±0.0**	**0.6±0.1**	**1.1±0.1**
i16:1	0.5±0.5	-	-	-	-	-	-	-	-	-	-	-
16:1ω7	4.9±0.5	17.7±0.6	11.1±2.1	6.7±0.2	5.6±0.7	7.5±0.7	10.6±1.3	6.2±1.1	10.2±0.7	6.7±0.0	4.6±0.1	8.1±0.9
16:1 ω5	**-**	**0.5±0.1**	**5.9±1.3**	**-**	**-**	**0.5±0.1**	**0.3±0.5**	**-**	**-**	**1.6±0.1**	**0.6±0.0**	**1.6±0.1**
i16:0	4.2±0.5	-	-	2.8±0.2	2.0±0.3	2.7±0.2	1.2±0.3	1.4±0.6	1.2±0.3	-	0.5±0.1	0.4±0.3
16:0	11.9±2.3	33.9±0.6	28.9±3.1	19.8±0.4	19.8±0.5	17.4±0.7	21.4±1.4	26.4±1.0	23.2±1.3	18.1±1.4	22.1±1.0	21.8±0.1
17:1ω9	-	-	-	0.7±0.2	1.5±0.5	0.9±0.2	-	-	-	-	0.4±0.1	-
17:1ω8	-	-	-	0.7±0.1	-	-	1.3±0.7	1.9±0.6	0.9±0.2	0.5±0.1	0.4±0.1	-
i17:0	**5.3±1.3**	**-**	**-**	**5.2±0.1**	**3.7±0.5**	**3.5±0.3**	**1.7±0.9**	**-**	**1.1±0.2**	**1.6±0.4**	**1.4±0.4**	**0.9±0.1**
a17:0	0.6±0.5	-	-	0.9±0.1	0.8±0.2	0.8±0.1	0.5±0.6	0.3±0.6	0.9±0.2	-	0.4±0.1	-
cy17:0	**-**	**16.9±0.2**	**31.4±3.2**	**0.5±0.1**	**0.9±0.1**	**1.3±0.0**	**6.3±0.9**	**5.7±0.5**	**3.2±0.9**	**14.3±1.0**	**12.0±0.9**	**14.7±0.2**
17:0	-	0.8±0.2	-	-	-	-	1.0±0.3	1.5±0.6	1.1±0.4	-	-	-
18:3ω6.9.12	-	-	-	-	-	0.4±0.1	1.6±0.3	1.5±0.1	1.5±0.3	0.6±0.1	-	-
18:2ω6.9	1.9±0.6	-	-	3.7±0.3	4.1±0.5	12.0±0.7	4.7±1.1	3.4±0.6	11.7±1.2	5.3±1.0	2.0±0.2	10.8±0.3
18:1ω9	0.3±0.5	-	-	28.0±0.6	32.2±0.3	30.4±1.0	20.9±1.1	25.1±3.6	23.3±2.8	26.2±0.9	29.4±1.2	25.0±1.4
18:1ω7	-	15.9±1.0	5.5±0.7	5.6±0.3	7.6±1.3	3.1±0.4	9.2±1.1	8.8±0.3	5.6±1.2	6.0±0.5	8.1±0.3	5.7±0.2
18:0	1.5±0.5	0.6±0.0	-	10.4±0.6	6.2±1.4	4.9±0.5	8.7±1.3	14.8±1.5	6.3±0.4	8.4±0.6	8.4±1.0	4.5±0.6
cy19:0	-	0.4±0.1	0.5±0.5	0.6±0.0	0.2±0.3	-	0.5±0.5	0.2±0.3	-	1.5±0.2	2.5±0.7	0.9±0.2
20:4ω6.9.12.15	-	-	-	1.2±0.0	3.2±0.8	2.4±0.1	3.9±0.4	1.9±0.5	3.2±0.4	3.1±1.4	0.7±0.0	1.9±0.1
20:2ω6.9	-	-	-	1.0±0.1	-	0.6±0.1	1.3±0.3	-	0.3±0.5	1.0±0.1	0.2±0.4	0.5±0.1
20:1ω9	-	-	-	-	-	-	-	-	-	-	1.1±0.1	-
20:0	-	-	-	0.7±0.3	-	0.3±0.1	-	-	0.3±0.6	0.4±0.1	0.6±0.1	-

Table 4.2:

Fatty acid pattern (means in % of total FAs ± s.d.) of the bacteria *Bacillus megaterium*, *Enterobacter aerogenes* and *Pseudomonas putida* and the neutral lipid fatty acid pattern (means in % of total FAs) of the Collembola *Folsomia candida*, *Heteromurus nitidus* and *Protaphorura fimata* fed with these bacteria. Marker FAs for bacterial diet are in bold. FAs which were not detected are maked with -.

i16:0 and trace amounts of a17:0. With *P. putida* or *E. aerogenes* as diet 3.3 to 6.9% of cy17:0 were present (except *P. fimata* fed on *E. aerogenes*, with none).

4.4 Discussion

Collembola are regarded as food generalists, feeding on a wide range of food resources (Parkinson, 1988; Rusek, 1998; Scheu and Setälä, 2002). Only few studies investigated bacteria as food source for Collembola. Thimm et al. (1998) observed that *F. candida* feeds on *P. putida*. Bakonyi et al. (1995) found *F. candida* and *Sinella coeca* to prefer the bacterium *Micrococcus luteus* over the fungus *Trichoderma viride* in a food choice experiment. This bacterial consumption of Collembola may alter the composition of soil microbial community by selective gut passage of bacteria (Thimm et al., 1998; Hoffmann et al., 1999). However, bacteria are not seen as important for nutrition of Collembola species, hitherto usually assumed to feed on fungi (Rusek, 1998). Here we showed, that Collembola fed and reproduced on all offered bacterial diets.

Total dry weight of Collembola maintained with *E. aerogenes* was significantly lower than in Collembola fed with *B. megaterium* and *P. putida*, suggesting that *E. aerogenes* is of low food quality. The NLFA/PLFA ratio was used by Bååth (2003) to measure the physiological status of soil fungi, with a higher ratio indicating an increase in storage fat likely due to excess carbon in the resource consumed. *F. candida* showed no difference in NLFA/PLFA ratio with food source, whereas *H. nitidus* and *P. fimata* had a lower ratio when reared on *E. aerogenes* compared with *P. putida* and *B. megaterium*.

When lipids are synthesised from dietary carbohydrate the isotopic composition of the synthesised fatty acid is strongly depleted in ^{13}C due to the fractionation during the oxidation of pyruvate to acetyl coenzyme A (Gannes et al., 1998; McCutchan et al., 2003). Lipids can be depleted by up to 10‰ relative to other animal tissues (Tieszen and Boutton, 1988). Therefore, the metabolic status of an animal (i.e. the amount of storage fat) affects the whole body δ^{13}C signature (Focken and Becker, 1998; Gearing, 1991). Collembola were depleted in 13C when fed with *B. megaterium* or *P. putida*, but slightly depleted or enriched when fed with *E. aerogenes*. Differences are likely due to a higher amount of storage fat in Collembola reared on high quality diet. This supports the findings in body dry weight, C/N ratio and NLFA/PLFA ratio.

The fractionation of nitrogen isotopes was influenced significantly by the food source and was highest in Collembola fed with *E. aerogenes*, intermediate with *B. megaterium* and lowest with *P. putida*. High fractionation rates for ^{15}N indicate that animals live on poor food sources or starve (Hobson et al., 1993; Adams and Sterner, 2000). This has also been shown for Collembola, where starving animals were enriched in ^{15}N (Haubert et al., 2005, see Chapter 3). In sum, these results indicate that the food quality of the different bacteria for Collembola is highest for *P. putida*, intermediate for *B. megaterium* and low for *E. aerogenes*.

The composition of NLFAs in the deposit fat results from different processes including the storage of dietary lipids, de novo synthesis, degradation and subsequent release

for mobilization to sites where they are metabolised (Beenakkers et al., 1985). Ruess et al. (2002, 2004, 2005a) reported trophic transfer of FAs from fungal and nematode diet predominantly into the neutral lipids of Collembola. This suggests that the lipid components of Collembola may reflect the pattern of their food source. Ruess et al. (2002, 2004, 2005a) reported trophic transfer of FAs from fungal and nematode diet predominantly into the neutral lipids of Collembola. This is also supported by results of the present study where, depending on the bacterial food source, NLFA profiles of Collembola differed significantly indicating dietary routing of FAs. In contrast, PLFAs were only little affected by diet. The pattern of PLFAs, which are structurally and functionally involved in biomembranes, is individually arranged in specific tissues and controlled at the cellular level (Stanley-Samuelson et al., 1988).

The present study identified specific FAs reflecting a bacterial diet in Collembola. Methyl-branched (iso, anteiso) acids are characteristic for gram-positive, and cyclopropyl rings are predominantly found in gram-negative bacteria (Welch, 1991; Zelles, 1999) and corresponding FA biomarkers occurred in NLFA profiles of Collembola (Table 4.2). Bacteria derived FAs were not present in the profiles of Collembola feeding on other diets such as fungi or leaves (Haubert et al., 2004, see Chapter 2; Ruess et al., 2004, 2005a,b). However, methyl-branched FAs were observed in considerable amounts on nematode diet (Chamberlain et al., 2005). As this study was performed with bacterial-feeding nematodes, this suggests the nematodes themselves have taken up the signal FA from their bacterial diet. Presumably, Collembola do not have the enzymatic capabilities to synthesise these FAs. This strongly suggests uptake from the bacterial food source and dietary routing into neutral lipids. Bacterial marker FAs (Table 4.2) include i14:0 i15:0, a15:0 and i17:0 for gram-positives sources such as *B. megaterium*. Marker FAs for gram-negatives were cy17:0 for *E. aerogenes*, and 16:1ω5 and cy17:0 for *P. putida*. The occurrence of these markers in neutral lipids indicates incorporation into the consumer's tissue, as FAs from bacteria originating from the gut of Collembola would be predominantly polar (Ratledge and Wilkinson, 1988).

However, the most abundant FAs in bacteria are not necessarily the best marker FAs for tracing food resource. For example the most abundant FA in *B. megaterium* was i13:0 (27.9%), which was only present in trace amounts in Collembola (0 - 0.6%). Obviously, some dietary FAs are degraded to acetly coemzyme A and catabolised, whereas others are subjected to dietary routing into storage fat. The underlying mechanisms are not yet clear.

4.5 Conclusions

The present experiment showed that bacteria contain several FAs that are not routinely biosynthesised by Collembola and therefore can be used as biomarkers reflecting the diet of the consumer. Estimates of NLFA/PLFA ratio, body weight, δ^{13}C and δ^{15}N gave additional information on the metabolic status of the Collembola and the food quality of the diet. Combined analysis of fatty acids and stable isotopes is a useful tool to analyse

the diet of Collembola and other soil animals for opening the black box of decomposer–resource interrelationships.

Chapter 5

Effects of temperature and life stage on the fatty acid composition of Collembola

Abstract

Fatty acid (FA) analysis is used as a promising tool to investigate trophic interactions in soil food webs. The FA profile of neutral lipids in consumers is affected by the diet, and the occurrence and amount of certain FAs indicate feeding strategies. We investigated the lipid composition of the Collembola *Folsomia candida*, *Heteromurus nitidus* and *Protaphorura fimata* with the fungus *Chaetomium globosum* as food source. The impact of environmental temperature and life stage was assessed, with special respect to linoleic acid (18:2ω6,9) the marker FA for fungal feeding.

In all Collembola species the ratio of C16/C18 in neutral lipid fatty acids (NLFAs) increased with decreasing temperature. In the NLFAs of *F. candida* and *H. nitidus* the Unsaturation Index (*UI*) and the amount of 18:2ω6,9 declined with temperature, whereas in *P. fimata* effects were vice versa. The composition of phospholipid fatty acids (PLFAs) differed between species, but was little affected by temperature. The degree of unsaturation in NLFAs increased with the age of Collembola, mainly due to higher amounts of 18:2ω6,9 and 16:1ω7, and a lower proportion of 18:1ω9.

The biomarker linoleic acid represented over 20% of FAs in all fungal feeding Collembola. Despite considerable influence of temperature and life stage on its proportion, the amount was always higher than in individuals reared on other diets. Therefore, linoleic acid can serve as indicator for fungal feeding independent of physiological variations in Collembola caused by ambient temperature or life stage.

5.1 Introduction

Fatty acid patterns have been frequently used to investigate food webs in aquatic systems (Ederington et al., 1995; Meziane et al., 1997; Navarrete, 2000). Only recently this approach was applied in soil food webs (e.g. Chen et al., 2001; Chamberlain et al. 2005; Ruess et al., 2002, 2004). Several studies investigated the feeding strategies of Collembola, a widespread and abundant group of soil animals, which play an important role in decomposition processes (Visser, 1985). Laboratory experiments showed that specific FAs are typically found in the neutral lipid fatty acids (NLFAs) of consumers, when reared on a certain diet (Haubert et al., 2006, see Chapter 4; Ruess et al., 2004). For bacterial feeding specific marker FAs indicate consumption of gram-positive (i14:0, i15:0, a15:0, i17:0) or gram-negative (cy17:0) bacteria (Haubert et al., 2006, see Chapter 4). The FA 20:1ω9 was only present in Collembola feeding on nematodes, whereas on fungal diet they contained a higher proportion of 18:2ω6,9 and on leaf diet a higher proportion of 18:1ω9 (Ruess et al., 2005b).

However, not only diet can influence the FA composition of an animal (Fernando-Warnakulasuriya et al., 1988; Howard and Stanley-Samuelson, 1996). The relative abundance of FAs in insects is also determined by the specific type of biosynthetic pathway of the given species (Hanson et al., 1985; Ghioni et al., 1996), life stage (Ogg and Stanley-Samuelson, 1992; Sayah et al, 1997), and environmental conditions (Joanisse and Storey, 1996). Especially thermal adaptation is a known factor in alterations of fatty acid composition (Hazel, 1995), but most studies deal with influence on phospholipid fatty acids (PLFAs). They are the main component of cell membranes and their composition is important to maintain membrane fluidity and subsequently membrane transport and cell functionality. Generally, the amount of unsaturated PLFAs in animals increases with declining environmental temperature (Hazel, 1995; Abu Hatab and Gaugler, 1997). In contrast NLFAs have an important role as energy reserves and are closely related to nutritional requirements and metabolism (Stanley-Samuelson et al., 1988). The composition of NLFAs in the fat body results from different processes including the storage of dietary lipids, de novo synthesis, degradation and subsequent release for mobilisation to sites where they are metabolised (Beenakkers et al., 1985). Due to their use to investigate trophic relationships (Haubert et al., 2006, see Chapter 4; Ruess et al., 2004, 2005b), it is important to assess effects which may influence the NLFA pattern.

Collembola are known to feed on a large variety of food sources (Rusek, 1998), but fungi are generally regarded as the most important diet (Chen et al., 1995; Klironomos and Kendrick, 1995). The biomarker FA linoleic acid (18:2ω6,9) is found in high amounts in Collembola feeding on fungi, but is also found in Collembola feeding on other diets, even though in lower amount (Haubert et al., 2006, see Chapter 4; Chamberlain et al., 2005, Ruess et al., 2005b). Physiological changes in Collembola metabolism may affect its occurrence, as well as related precursors and descendants. Linoleic acid is the precursor for eicosanoids, which play an important role in insect physiology. They influence reproduction, mediate cellular immune response and are involved in temperature regulation

(Stanley-Samuelson and Nelson, 1993; Toolson et al., 1994). In addition, the amount of linoleic acid may be altered to maintain membrane fluidity at different environmental temperatures. We have analysed the influence of temperature and life stage on the lipid pattern in three Collembola species (*Protaphorura fimata*, *Heteromurus nitidus*, *Folsomia candida*) reared on fungal diet focusing on linoleic acid.

5.2 Materials and Methods

5.2.1 Fungi

The soil decomposer fungus *Chaetomium globosum* Kunze was cultivated at 10°C on Potato dextrose agar (PDA, Merck, Darmstadt). As food source for Collembola round pieces of fungal mats (diameter 10 mm) were cut out of the agar cultures under sterile conditions and offered to animals. For analysis of fungal FA composition *C. globosum* was grown on PDA covered by a membrane filter (Millipore, 0.8 μm). Cultures were kept at 5, 10 or 15°C for 3 days before harvest to simulate the incubation conditions for fungi in the feeding experiment. To gain fungal biomass membrane filters were stripped of the agar with the adhering fungal mats. Hyphae were scrapped from the filter with a sterile scalpel and frozen at -20°C until analysis.

5.2.2 Collembola

The Collembola species *Protaphorura fimata* (Gisin, 1952), *Heteromurus nitidus* (Templeton 1835) and *Folsomia candida* (Willem 1902) were taken from laboratory cultures fed with bakers yeast. Specimens were kept in plastic vessels (diameter 7 cm, height 4.5 cm) with a layer of plaster mixed with activated charcoal (2:1) at the bottom. Each vessel contained ten individuals. This low density was chosen to avoid cannibalism due to crowding. Vessels were kept in darkness and under moist conditions with distilled water. Eggs, pellets and exuvia were removed once a week. To investigate the effect of temperature Collembola were incubated at 5, 10 and 15°C. Animals were fed with fungal diet ad libitum, thereby the fungal food source was renewed three times a week. After six weeks Collembola were sampled destructively (n=3) and frozen at -20°C until analysis.

To study the influence of different life stages eggs of *P. fimata* were kept in plastic vessels until hatching. Collembola hatched within one week were joined into the same life stage group. Specimens were fed with *C. globosum* for 4, 8 and 12 weeks after hatching. Three replicates (with 30 individuals each) per developmental stage were performed and harvested destructively. Collembola were frozen at -20°C until analysis.

5.2.3 Analysis of fatty acid patterns

Whole cellular lipids of fungi were extracted, whereas lipids of Collembola were divided into NLFA and PLFA fractions. Collembolan lipids were extracted by shaking in 5 ml single phase extraction solvent (chloroform/methanol/0.05 M phosphate buffer (pH 7.4) 1:2:0.8) overnight. The solvent was then transferred to new tubes and samples were re-extracted by shaking for 2-3 h with additional 2.5 ml. Extraction solvents of both steps were combined, 0.8 ml distilled water and 0.8 ml $CHCl_3$ were added and samples centrifuged at 1500 rpm for 5 min. Samples were allowed to stand and separate. Then the top two phases were removed and the chloroform fraction of each sample was transferred to a silica acid column (0.5 g silicic acid, mesh size 100-200 μm). Lipids were eluted with 5 ml chloroform (neutral lipids), 8 ml acetone (glycolipids) and 5 ml methanol (phospholipids). Neutral lipids and phospholipids were analysed further. The chloroform and methanol fraction was reduced by evaporation (50°C, vacuum 200 hPa) in a Labconco RapidVap (Labconco Corporation, Kansas City).

Chloroform and methanol fractions of the Collembola samples and total fungal biomass were saponified and methylated following the procedures given for the Sherlock Microbial Identification System (MIDI Inc., Newark, Del.). Saponification of lipids was conducted in a sodium hydroxide-methanol solution (45 g sodium hydroxide, 150 ml methanol, 150 ml distilled water) at 100°C for 30 min, followed by acid methanolysis in HCl-methanol (325 ml 6.0 N hydrochloric acid, 275 ml methyl alcohol) at 80°C for 10 min. The fatty acid methylesters were extracted into hexane-methyl tertiary butyl ether (1:1) and washed with aqueous NaOH (10.8 g sodium hydroxide, 900 ml distilled water). The lipid-containing phase was then transferred to test tubes and stored at -20°C until analysis.

Fatty acid methyl esters were analysed by gas chromatography (GC) using the Sherlock Microbial Identification System (MIDI Inc.) consisting of a Hewlett Packard 5890 Series II gas chromatograph and flame ionisation detector equipped with an HP Ultra 2 phenyl methyl silicone fused capillary column (25 m $\times$ 0.2 mm i.d., film thickness 0.33 μm), an automated sampler and computer with associated software (Sherlock Pattern Recognition Software, MIDI). The fatty acid methyl esters were identified on the basis of their retention times and quantified. To verify correct identification of fatty acids methyl esters (chain length and saturation) all samples were analysed by GC-mass spectrometry using an Agilent Series 6890 GC System and 5973 Mass Selective Detector, equipped with a HP5MS capillary column (30 m $\times$ 0.25 mm i.d., film thickness 0.25 μm).

The fluidity of lipids is expressed as the fatty acid Unsaturation Index (UI). This was calculated as follows:

$$UI = \frac{(\text{C:1} \cdot 1) + (\text{C:2} \cdot 2) + (\text{C:3} \cdot 3) + (\text{C:4} \cdot 4)}{100}$$

where C:1, C:2, C:3 and C:4 represents the proportion (%) of fatty acids with 1, 2, 3, and 4 double bonds, respectively.

5.2.4 Statistical analysis

Differences in fatty acid profiles of fungi and Collembola were analysed using ANOVA. If significant effects were suggested, means were compared with Tukey's honestly significant difference test. Statistical analyses were performed using SAS (SAS Institute Inc., Cary, USA).

5.3 Results

5.3.1 Effects of temperature

Fungi

Major cellular lipids of *C. globosum* were palmitic (16:0; 18-20%), oleic (18:1ω9; 29-32%) and linoleic (18:2ω6,9; 44-46%) acid. Additionally, 14:0, 16:1ω7, 17:0 and 18:0 were present in proprotions between 0.5 and 4.7%. The FA profile of *C. globosum* was not significantly affected by temperature within 3 days of incubation at 5, 10 and 15°C (Table 5.1).

	5°C	10°C	15°C	$F_{4,2}$	P
14:0	0.5±0.2	0.6±0.1	0.5±0.1	0.82	0.5020
16:0	18.9±0.7	20.3±4.2	18.2±0.4	0.46	0.6584
16:1ω7	1.0±0.2	1.1±0.4	1.0±0.1	0.06	0.9432
17:0	0.8±0.1	1.0±0.3	0.9±0.2	0.81	0.5077
18:0	4.7±1.2	1.8±1.7	3.8±0.1	3.16	0.1502
18:1ω9	30.4±1.4	29.0±2.9	31.8±2.0	0.99	0.4483
18:2ω6,9	43.8±1.2	46.2±5.5	43.8±2.2	0.44	0.6702

Table 5.1:
Effects of incubation temperature on the proportion of fatty acids (in % ± s.d.) in cellular lipids of the fungus *Chaetomium globosum* after 3 days at 5, 10 or 15°C.

Collembola

Ambient temperature significantly affected body weight of Collembola ($F_{2,18}$=20.66, P<0.0001, Fig. 5.1). Irrespective of species individuals kept at 5°C had a lower fresh weight (340 - 437 μg) than Collembola maintained at 10 or 15 °C (485 - 557 μg).

Dominant NLFAs of Collembola were similar in *P. fimata*, *H. nitidus*, and *F. candida*, but differed in their amount due to species (Table 5.2). Major FAs were 16:0 (17.3-25.7%), 16:1ω7 (2.0-7.5%), 18:0 (2.0-7.2%), 18:1ω9 (45.9-51.3%) and 18:2ω6,9 (12.6-24.8%). Temperature had a significant influence on the proportion of FAs, except for oleic acid. Palmitic and palmitoleic acid (16:1ω7) decreased with elevated temperature in all three species ($F_{2,18}$=4.75, P=0.0221, $F_{2,18}$=14.73, P=0.0002, respectively). The proportion of linoleic acid in *H. nitidus* kept at 5°C was about half compared to incubation at

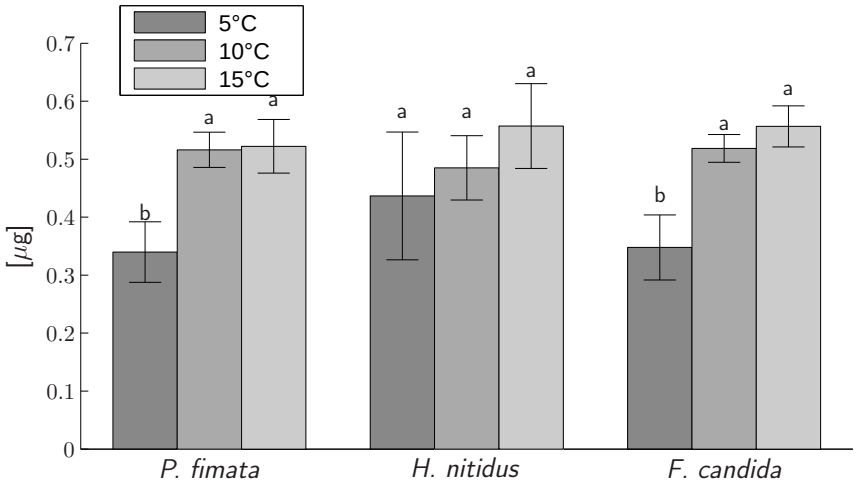


Figure 5.1:
Fresh weight (μg) of 3 Collembola species (*Protaphorura fimata*, *Heteromurus nitidus*, *Folsomia candida*) fed with *Chaetomium globosum* under different temperature conditions (5, 10, 15°C). Bars (± s.d.) sharing the same letters are not significantly different (Tukey's HSD, $P<0.05$).

10 and 15°C ($F_{2,6}$=7.69, P=0.0221). Stearic acid (18:0) was more abundant in *P. fimata* at 15°C with 4.4%, than at 5 and 10 °C with only 2.0 and 2.4%, respectively ($F_{2,6}$=20.48, P=0.0021).

The ratio C16/C18 in neutral lipids decreased significantly with increasing temperature in all Collembola ($F_{2,18}$=11.93, P=0.0005). *H. nitidus* always had the lowest Unsaturation Index (*UI*) of NLFAs among species ($F_{2,18}$=19.9, $P<0.0001$). The *UI* in *P. fimata* increased from 0.97 to 1.00 with declining temperature, whereas in *H. nitidus* and *F. candida* the *UI* decreased when maintained at 5°C compared to 15°C (0.92 to 0.83, 1.00 to 0.96, respectively).

Phospholipids of *H. nitidus* could not be analysed due to insufficient biomass and a high mortality at 5°C. Major PLFAs of *F. candida*, and *P. fimata* were 16:0 (24.4 - 31.2%), 18:0 (7.5 - 23.9%), 18:1ω9 (17.9 - 34.8%) and 18:2ω6,9 (28.8 - 34.9%) (Table 5.3). No significant influence of temperature on the PLFAs was observed, whereas the composition of FAs varied with species. In *P. fimata* 18:0 was significantly less and 18:2ω6,9 more abundant than in *F. candida* ($F_{1,6}$=60.7, P=0.0002; $F_{1,6}$=69.4, P=0.0002; respectively). This corresponded to the *UI*, which was higher in *P. fimata* (0.97 - 1.03) than in *F. candida* (0.69 - 0.75; $F_{1,6}$= 70.5; P=0.0002). The C16/C18 ratio decreased with increasing temperature in both species, most pronounced in *F. candida* (0.45 to 0.33) compared to *P. fimata* (0.32 to 0.28).

5.3.2 Influence of life stage

The life stage of Collembola affected the body mass of specimens. *P. fimata* at an age of 4 weeks had a lower fresh weight (159 ± 36 μg) compared to 8 and 12 weeks old individuals (297 ± 63 μg and 262 ± 129 μg, respectively, data not presented). Additionally, the proportion of dominant NLFAs changed with developmental stage. The amount of 16:1ω7 and

Collembola	*P. fimata*			*H. nitidus*			*F. candida*			ANOVA		
Temperature [°C]	5	10	15	5	10	15	5	10	15	C	T	C×T
16:0	22.3±0.6	22.4±0.6	20.5±0.8	25.7±1.9 a	22.0±0.6 b	24.9±1.2 ab	20.2±1.3	21.2±2.3	17.3±0.7	***	*	**
16:1ω7	4.5±1.7	3.5±1.3	2.0±0.5	7.4±2.2	5.0±1.2	3.4±2.0	7.5±1.1 a	4.7±1.1 b	3.1±1.1 b	*	***	
18:0	2.0±0.4 b	2.4±0.2 b	4.4±0.7 a	4.2±0.7	3.2±0.2	3.7±0.2	5.2±1.9	4.9±0.2	7.2±1.7	***	**	
18:1ω9	47.0±2.4	49.3±3.3	51.3±0.1	50.2±4.1	48.0±3.3	47.1±1.2	46±2.9	45.9±2.2	47.5±0.5			
18:2ω6,9	24.2±0.3	22.4±2.3	21.8±0.9	12.6±3.0 b	21.8±4.0 a	20.9±2.1 a	21.2±1.0 b	23.4±1.2 ab	24.8±1.6 a	***	**	**
C16:C18	0.37±0.04	0.35±0.03	0.29±0.02	0.50±0.09	0.37±0.01	0.39±0.03	0.38±0.05	0.35±0.06	0.26±0.01	***	***	
UI	1.00±0.01	0.98±0.02	0.97±0.01	0.83±0.04	0.97±0.04	0.92±0.03	0.96±0.01	0.97±0.03	1.00±0.04	***	**	***

Table 5.2:

Major neutral lipid fatty acids (in % ± s.d.) of collembola *Protaphorura fimata*, *Heteromurus nitidus* and *Folsomia candida* maintained at three different temperatures (5, 10, 15 °C). ANOVA indicating significant effects of Collembola (C), temperature (T) and interactions (C×T), with *, **, *** with $P < 0.05, 0.01, 0.001$.*UI* - Unsaturation Index

Collembola	*P. fimata*					*F. candida*			ANOVA
Temperature [°C]	5		10		15	5	10	15	C
16:0	24.4	-	24.5	-	21.6±1.6	31.2±2.3	26.3±6.9	24.7±1.2	
18:0	11.7	-	7.5	-	10.7±3.7	18.5±1.0	21.9±3.6	23.9±2.1	***
18:1ω9	30.9	-	34.8	-	32.9±2.1	18.6±1.5	23.0±2.3	17.9±4.4	
18:2ω6,9	33.0	-	33.2	-	34.9±3.7	31.7±1.9	28.8±2.2	33.6±1.0	***
C16:C18	0.32	-	0.32	-	0.28±0.03	0.45±0.05	0.37±0.13	0.33±0.02	
UI	0.97	-	1.01	-	1.03±0.05	0.69±0.05	0.75±0.07	0.69±0.02	***

Table 5.3:
Major phospholipid fatty acids (in % ± s.d.) of the Collembola *Protaphorura fimata* and *Folsomia candida* maintained at 3 different temperatures (5, 10, 15 °C). *UI* – Unsaturation Index. ANOVA indicating significant effects of Collembola species (C) with *** with $P < 0.001$.

18:2ω6,9 increased ($F_{2,5}$=12.04, P=0.0123; $F_{2,5}$=9.37, P=0.0204; respectively), whereas the frequency of 18:1ω9 decreased with increasing age ($F_{2,5}$=5.74, P=0.0507, Fig. 5.2). The C16/C18 ratio ranged between 0.31 and 0.33 (data not presented) and was not influenced by life stage. The *UI* tended to increase from 0.94 to 1.08 in older Collembola ($F_{2,5}$=4.94, P=0.0655, data not presented).

5.4 Discussion

5.4.1 Temperature

Mean soil temperature in 5-10 cm depth is 8-10°C for temperate regions and depends more on air temperature, the closer the soil layer is to the surface. The incubation temperature used in the present experiments therefore represent a range of naturally occurring environmental conditions for Collembola inhabiting temperate soils. Investigations on temperature response in Collembola were predominantly made on *F. candida*, indicating this species to develop well between 13 and 21°C (Martikainen and Rantalainen, 1999; Fountain and Hopkin, 2005). Johnson and Wellington (1980) observed a 50% reduction in growth rate when temperature was reduced from 16 to 8°C, but only a minor increase with temperature elevation from 16 to 24°C. Snider and Butcher (1973) found about 24% difference in reproduction of *F. candida* between 15 and 21°C. In our experiment Collembola cultured at 5°C had a lower body weight than Collembola kept at 10 or 15°C. This suggests optimal temperature conditions for development of *P. fimata*, *H. nitidus* and *F. candida* well above 5°C.

H. nitidus was the most temperature sensitive species in our experiment and had a high mortality at 5°C. The natural habitat of this epedaphic Collembola ranges from temperate to subtropical regions (http://www.collembola.org). Bayley et al. (2001) recorded a high mortality for *F. candida* at 2 - 3°C. However, we observed no deleterious effects on *F. candida* at 5°C. This Collembola species occurs in all climatic regions from the

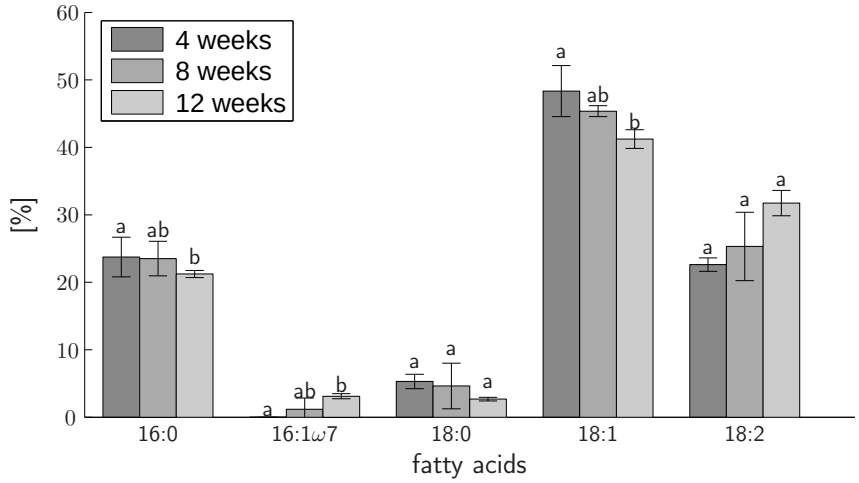


Figure 5.2:
Amount of major neutral lipid fatty acids of 4, 8 and 12 week old *Protaphorura fimata* fed with *Chaetomium globosum*. Bars (± s.d.) sharing the same letters are not significantly different (Tukey's HSD, $P<0.05$).

Antarctic to the tropics and can adapt life history strategy to environmental conditions. *F. candida* is an r-strategist under good conditions and switches towards K-strategy under poor conditions (van Amelsvoort and Usher, 1989). Also, no mortality was observed at 5°C for the euedaphic *P. fimata* which occupies habitats from polar to the temperate regions.

The ambient temperature did not influence the fatty acid composition of the fungal diet *C. globosum* within 3 days, the maximum incubation period with Collembola. The altered FA composition of Collembola due to temperature regime in the experiment therefore originates from changes in animal metabolism and is not related to differences in the food source.

Animals adapt to variations in temperature conditions mainly by altering the fatty acid composition of membranes. The changes during cold acclimation to maintain membrane viscosity and function are termed homeoviscous adaptation (Hazel, 1995). Two major metabolic compensation mechanisms exist. First, short-chain FAs result in higher membrane fluidity than long-chain FAs, leading to temperature induced changes in the ratio of C16 to C18. Second, unsaturated FAs result in more fluidity than saturated FAs, and the Unsaturation Index (*UI*) therefore increases with lower temperature (Hazel and Williams, 1990). Abu Hatab et al. (1997) analysed the influence of temperature on both PLFA and NLFA in nematodes, and found an increase in saturated FAs with increasing temperature. Similar shifts were found for lizards (Shen et al., 2005) and fish (Jobling and Bendiksen, 2003). However, most studies on temperature adaptations in organisms refer to alterations in PLFAs (e.g. Cuculescu et al. 1995; Tsugawa and Lagerspetz, 1990), whereas changes in NLFAs are not well documented. For the use of FAs as biomarkers in food chains, changes in NLFAs are important, as dietary FAs are predominantly incorporated into deposit fat of the consumers (Haubert et al., 2006, see Chapter 4; Ruess et al.,

2004)

Fluidity in storage fat is maintained at lower temperatures to permit accessibility of enzymes to energy reserves (Gordon et al., 1979, Jagdale and Gordon, 1997). In our experiments the C16/C18 ratio of NLFAs increased with decreasing temperature. Individuals kept at lower environmental temperatures contained higher proportions of palmitic acid (16:0). Similar observations are reported from *Drosophila* spp. (Ohtsu et al., 1998). In contrast to the C16/C18 ratio, the *UI* of NLFAs increased with temperature in *F. candida* and *H. nitidus*, but decreased in *P. fimata*, mainly due to corresponding proportions of the unsaturated FA linoleic acid. Oleic acid (18:1ω9) did not respond to temperature at all. These results are surprising, as adaptation to lower temperature by shifting from more saturated to more unsaturated FAs is a widespread phenomenon in poikilothermes. For instance in nematodes the *UI* of NLFA and PLFA increased with decreasing temperature (Jagdale and Gordon, 1997). However, Petersen and Holmstrup (2000) found an increase in *UI* of whole cell fatty acids of *Lumbricus rubellus* from 1.4 to 1.6 when kept at 0°C in comparison to 20°C. In our experiments only *P. fimata* showed an increase in *UI* with cold acclimation, whereas all species displayed a distinct increase in the C16/C18 ratio. This indicates that Collembola change the fluidity in their NLFAs mainly by alterations in FA chain length and not by degree in saturation.

In contrast to other studies which found the degree of unsaturation in PLFAs to vary inversely with growth temperature in poikilothermic animals (Fodor et al., 1994; Abu Hatab and Gaugler, 1997; Petersen and Holmstrupp, 2000; Jobling and Bendikson, 2003; Overgaard et al., 2005) the impact of ambient temperature on the PLFA pattern of Collembola in our experiment was minor. Declining temperature led to a shift towards short chain FAs which increases the fluidity of membranes. However, these effects were not significant and the impact of Collembola species on the composition of PLFAs was much more pronounced than temperature effects.

The proportion of 18:2ω6,9 in NLFAs, the marker FA for fungal feeding in Collembola (Ruess et al., 2005b), was over 20% in all species and at all incubation temperatures. This is well above the amount in Collembola feeding on bacteria (2.0-12.0%, Haubert et al., 2006, see Chapter 4), leaves (13.9%) and nematodes (13.6%, Ruess et al. 2005b), or yeast (2.0-12.0%, Chamberlain et al., 2005). Proportions below 20% occurred only in *H. nitidus* at 5°C (12%), where this species had a high mortality. Likely, the FA pattern at 5°C is not predominantly affected by the major diet ingested, but by altered metabolic conditions due to temperatures below the tolerated threshold. Suboptimal growth temperatures are reported to result in qualitative and quantitative changes in lipid composition of nematodes (Abu Hatab and Gaugaler, 1997). *H. nitidus* had the lowest *UI* at all temperatures, suggesting poorer adaptation to temperature decline than in *P. fimata* and *F. candida*. This may be due to different temperature optima in enzymes that synthesise unsaturated FAs, a fact known for eukaryotic organisms (Erwin, 1973). A comparable inability to adapt to decreasing temperatures was also found by Jagdale and Gordon (1997) in a nematode species inhabiting warmer regions which showed a poor survival at 5°C.

5.4.2 Life stage

Fatty acid profiles of many insects species change over the course of development (Stanley-Samuelson et al., 1988). In gypsy moth both NLFA and PLFA pattern changed with life stage (Stanley-Samuelson et al., 1992). In our experiments NLFA composition of *P. fimata*, especially the *UI*, was influenced by the age of the Collembola. The *UI* increased from 0.94 to 1.08 in older animals, mainly due to the higher proportion of 18:2ω6,9 and 16:1ω7 and the lower amount in 18:1ω9. Also Kamler et al. (2001) observed in fish that the level of some unsaturated fatty acids increased with age and size. Kattner and Krause (1987) found more long-chain unsaturated FAs in older stages of copepods.

The influence of age on C18 FAs is due to the fact that they are used as precursors for hormones (Stanley-Samuelson and Nelson, 1993). They are elongated to C20 PUFAs (polyunsaturated fatty acids), with arachidonic acid (20:4ω6,9,12,15) as precursor for eicosanoids. In invertebrates prostaglandins (a type of eicosanoids) are involved in reproduction, predominantly egg laying and moulting. They also modulate lipid mobilisation in insects and are mediators of hemocytic immunity (Stanley-Samuelson and Nelson, 1993). Due to the requirement of eicosanoids in reproduction processes, the life stage of the Collembola may affect the amount of FAs which are involved in the biosynthesis of C20 PUFAs such as 18:2ω6,9. In our experiments, we found only an insignificant increase in 18:2ω6,9 in neutral lipids with Collembola age. This suggests that developmental needs for precursor FAs of C20 PUFAs do not affect NLFA pattern and consequently the trophic marker linoleic acid.

5.5 Conclusions

Temperature and developmental stage affected the composition of NLFAs and PLFAs in Collembola. However, linoleic acid, the marker FA for fungal feeding in Collembola, was present in high proportions (over 20%) at all temperatures and stages (except *H. nitidus* at 5°C), and was more abundant than in Collembola feeding on other food sources. Therefore, high amounts of 18:2ω6,9 in NLFAs can serve as robust biomarker for fungal food in Collembola and presumably also other soil decomposer invertebrates.

Chapter 6

Carbon stable isotope ratios of fatty acids as a tool to investigate soil food webs - a field study using the natural label of C_3 and C_4 plants

Abstract

Lipid pattern and stable carbon isotope ratios ($^{13}C/^{12}C$) of fatty acids (FAs) were determined in consumers of the decomposer food web of agricultural fields planted with soybean (permanent C_3 crop $>$ 26 years) and maize (first vegetation period with C_4 crop), and managed either by conventional (CONMIN) or organic farming (bio-dynamic, BIODYN). We analysed FAs in microorganisms as primary decomposers, Collembola as secondary decomposers, and spiders as top predators. Differences in $\delta^{13}C$ of corresponding FAs in consumers and food sources were assessed. BIODYN farming led to an increase in microbial phospholipid fatty acids (PLFAs) and a more fungal dominated community. The $\delta^{13}C$ of microbial PLFAs reflected the change from C_3 to C_4 crop by a slight shift in $\delta^{13}C$ of +2‰, whereas the isotopic signal in FAs of Collembola was much more pronounced. In the euedaphic *Protaphorura fimata* the $\delta^{13}C$ signatures in maize fields exceeded that in soybean fields by up to 10 delta units, indicating close relationship between diet and vegetation cover. In the epedaphic *Orchesella villosa* $\delta^{13}C$ signatures shifted by only 4 delta units, suggesting incorporation of a wider food spectrum including carbon of former C_3 crop residues. Results of FA pattern and isotopic composition indicate that *P. fimata* consume predominantly fungi and *O. villosa* fed mainly on plant litter. This was comfirmed by corresponding $\delta^{13}C$ in marker FAs for consumption of fungi (18:2ω6,9) and plant tissue (18:1ω7) in Collembola and food resources. Comparison of $\delta^{13}C$ in FAs of the cursorial spider *Pardosa agrestis* and *O. villosa* revealed the latter as an important prey species at soybean fields. In contrast, the web-builing spider *Mangora acalypha* showed no relationship to Collembola prey.

6.1 Introduction

Over the last decade stable isotope analysis has routinely been used to investigate soil decomposer food webs (Scheu and Falca, 2000; Ponsard and Arditi, 2000; Schneider et al., 2004; Chahartaghi et al., 2005). Stable isotope ratios in organisms differ depending on their position in the food web and provide information on trophic level ($^{15}N/^{14}N$) or diet consumed ($^{13}C/^{12}C$) (Martin et al., 1992; Wada et al., 1993; Briones et al., 1999; Post, 2002). However, there are methodological drawbacks in using stable isotopes to define diets. Firstly, the need in isotopic variation among food sources to assign the consumed diet, and, secondly, the limitation by the number of available isotopes, i.e. when there are n food sources for the consumer, at least n-1 isotopes are necessary to resolve dietary dependencies (Matthews and Mazumder, 2004).

To increase the resolution for identifying trophic links the combination of gas-chromatography-combustion-isotope-ratio-monitoring-mass-spectrometry (GC-C-IRM-MS) with fatty acid (FA) analysis offers a valuable tool. Recently, FAs were proposed as trophic markers for feeding strategies of soil animals (Haubert et al., 2006, see Chapter 4; Ruess et al., 2004, 2005b; Chamberlain et al., 2005). Compound-specific analysis by GC-C-IRM-MS allows to monitor the respective $^{13}C/^{12}C$ ratios in FAs. This approach was successfully applied in marine ecosystems to distinguish between food chains based on either chemoautotroph bacteria or photoautotroph plants (MacAvoy et al., 2002; 2003). In soil $\delta^{13}C$ in FAs was used to link microbial community structure with activity. Isotopic labelling of biomarker FAs was employed to assign bacterial substrate usage (Abraham et al., 1998; Waldrop and Firestone, 2004; Pombo et al., 2005) or changes in bacterial and fungal activities (Arao, 1999). In sum, compound-specific analysis of biomarkers can identify microbes involved in specific processes and incorporate microorganisms into food web studies (Boschker et al., 1998; Boschker and Middelburg, 2002).

Generally, it is energetically more efficient to deposit FAs derived from food resources and to catabolise dietary carbohydrates directly, which results in the routing of dietary FAs into consumer tissues without degradation (Blem, 1976; Pond, 1981). Due to this direct incorporation the isotopic composition of these FAs is similar to the corresponding FAs in the diet. In contrast, FAs synthesised de novo reflect the $\delta^{13}C$ signatures of carbon catabolized from dietary macronutrients. To monitor the trophic transfer of FA biomarkers at natural $^{13}C/^{12}C$ abundance it is particularly tempting to use dietary constituents from C_3 and C_4 plants, which differ in their isotopic composition due to the different photosynthetic pathway. In plants using the C_3 pathway $\delta^{13}C$ signatures range between -18 and -36‰ and via the C_4 pathway between -7 and -18‰ (Tieszen and Boutton, 1989; Webb et al., 1998). In controlled animal feeding experiments, $^{13}C/^{12}C$ ratios measured in FAs provide an accurate representation of the routing and synthesis of dietary constituents form both C_3 and C_4 diets (Stott et al., 1997). Laboratory experiments on Collembola and nematodes have shown the power of this analysis in fungal based soil food webs (Chamberlain et al., 2004; Ruess et al., 2005a). However, field studies of decomposer communities have not hitherto been performed. We investigated the decomposer food

web in soils of the DOK (bio-**D**ynamic, bio-**O**rganic, **C**onventional) field experiment in Therwil, Switzerland (Besson and Niggli, 1991; Mäder et al., 2002). This agricultural field experiment, established in 1978, serves to examine effects of different farming systems, comprising different fertilization and plant protection schemes in the same crop rotation and with similar soil tillage. We sampled on soybean (C_3) fields, without any C_4 crop for at least 26 years, and fields where maize (C_4) was grown for the first time over the whole experimental period. Both crops were investigated in fields with either exclusive application of mineral fertiliser or manure compost and slurry in bio-dynamic treatments. The community investigated comprised microorganisms, Collembola and spiders. In agricultural soils Collembola represent a major component of the fauna with densities from 10,000 to 120,000 individuals per square meter (Larsen et al., 2004). Spiders are the most abundant generalist predators (Ferguson and Stilling, 1984; Young and Edwards, 1990), with wolf spiders (Lycosidae) as dominant cursorial spiders in crop systems of the temperate zone (Lang et al., 1999).

This study presents the first field survey of $^{13}C/^{12}C$ ratios in FAs of organisms inhabiting different trophic levels of the soil food web. It includes major functional groups, i.e. microorganisms as primary decomposers, euedaphic and epedaphic Collembola as secondary decomposers, and cursorial and web-building spiders as top predators. The FA composition, $\delta^{13}C$ signatures of FAs and difference of $\delta^{13}C$ signatures of FAs between consumer and diet were analysed. As agricultural practice may affect predator-prey interactions, conventional and organic farming sites were investigated. Our aim was to test the feasibility of compound-specific isotopic tracer studies to assess trophic interactions by using the $\delta^{13}C$ signal of C_3 and C_4 plants at the natural abundance level.

6.2 Materials and Methods

6.2.1 Field site

In 1978 the DOK field experiment comparing two organic and two conventional cropping systems was established at Therwil in the vicinity of Basel, Switzerland, by the Swiss Federal Research Station for Agroecology and Agriculture (Zürich-Reckenholz) in cooperation with the Research Institute of Organic Agriculture (Frick) (Besson and Niggli, 1991; Mäder et al., 2002). The soil at the site is a haplic luvisol (sL) on deep deposits of alluvial loess. The climate is humid and mild with a mean precipitation of 785 mm per year and an annual mean temperature of 9.5°C.

The cropping systems mainly differed in fertilization regime and plant protection. We investigated conventional (mineral, mimicking stockless farming; CONMIN) and organic (bio-dynamic; BIODYN) agricultural systems. CONMIN included NPK fertilization (118 kg N, 36 kg P, 242 kg K ha^{-1} y^{-1} from 1999 to 2005), mechanical and chemical weed control, chemical disease and insect control, and plant growth regulators. BIODYN comprised bio-dynamic treatment with composted farmyard manure and slurry (1.4 livestock units), mechanical weed control, indirect disease control, insect control with plant extracts

and biocontrol, and application of bio-dynamic preparations (e.g. cow horn, composting additives, decocts; see Reganold and Palmer, 1995). The BIODYN treatments received between 55 to 84% (100 kg N, 20 kg P, 194 kg K ha^{-1} y^{-1}) of the amount of nutrients applied to the CONMIN treatment. The soil pH was 5.1 and 6.1 in CONMIN and BIODYN treatments, respectively (Fließbach and Mäder, 2000).

The investigated systems harboured soybean or maize crop. A permanent C_3 vegetation crop was grown since the beginning of the experiment in 1978, i.e. maize was the first C_4 plant after at least 26 years. Maize fields had soybeans and a catch crop (rye) during winter as preceding crops, and before soybeans winter wheat followed by a rye catch crop was growing. The experiment is designed as a Latin square with individual field size of 5 × 20 m (for further details on site or management practice see Fließbach and Mäder (2000).

6.2.2 Sampling

The two farming systems (CONMIN, BIODYN) each comprising two crop plants (maize, soybean) and four field replicates were sampled in August 2004; for analysis of the microbial community soil cores of 3 cm diameter from 0-20 cm depth were taken in a distance of 10 cm to the respective crop row. The cores were kept cool at 2 - 4 °C until they were sieved through 2 mm mesh and analyzed. Plant tissue samples were collected from both soybean and maize plants. Mature green leaves were taken randomly from several plants. Roots were dug out with a spade and biomass harvested by cutting samples off with a knife.

For Collembola three soil samples (20 × 20 cm, 5 cm deep) per field were taken with a spade in August and November 2004. Collembola were extracted from soil using a high gradient extractor for 2 weeks (Macfadyen, 1961; Kempson et al., 1963). Animals were collected in water, separated daily under a dissecting microscope and determined. Collembola of the same taxon were bulked from August and November samplings in order to get sufficient biomass. *Orchesella villosa* (Linnaeus) and *Protaphorura fimata* (Gisin) occurred in ample amount for GC-C-IRM-MS analysis. Between 5 and 25 individuals were bulked per sample (n=4), transferred into methanol and stored at -20°C.

Two abundant spider taxa at the sites, *Mangora acalypha* (Walckenaer) and *Pardosa agrestis* (Westring), were collected from each field in August 2004 using an aspirator. *P. agrestis* comprised of adult and juvenil individuals. Spiders were determined and samples (n=4) consisting of 1 to 9 individuals were stored in methanol at -20°C until analysis.

6.2.3 Analysis of fatty acid pattern

Phospholipid fatty acids (PLFAs) of the microbial community were extracted from the soil and fractionated using the procedure described by Zelles and Bai (1993). Lipids were extracted by adding 250 ml methanol, 125 ml chloroform and 100 ml (including soil water) of phosphate buffer to fresh soil (corresponding to 50 g oven dry soil). Samples were shaken

for 2 h and another 125 ml of each water and chloroform were added for phase separation. After 24 h of phase separation the supernatant was decanted and the lower chloroform phase was filtered. The water free chloroform phase was reduced to 10 ml volume in a rotavator. Of the chloroform fraction 2.5 ml of each sample was dried under a nitrogen stream and then dissolved in 900 μl chloroform. Samples were transferred to a silica acid column (0.5 g silicic acid, 3 ml Varian Medical Systems, Palo Alto, California) and lipids were eluted with 5 ml chloroform (neutral lipids), 20 ml acetone (glycolipids) and 5 ml methanol (phospholipids). The phospholipid fraction was dried under a nitrogen stream. Each sample was dissolved in 1 ml methanol-toluene-solvent (1:1) and 30 μl internal Standard (5.77 mg methylnondecanoat in 25 ml isooctane) was added. Basic methanolysis of lipids was conducted in 1 ml 0.2 M methanolic KOH (2.8 g KOH in 250 ml methanol) with incubation for 15 min at 37°C. The fatty acid methyl esters (FAMEs) were extracted with 2 ml hexane-chloroform solvent (4:1), 0.3 ml 1 M acetic acid and 2 ml deionisated water. Samples were shaken and centrifuged at 2500 rpm for 10 min. The organic phase was transferred to new tubes and FAMEs were re-extracted with 2 ml hexane-chloroform solvent. Extraction solvents of both steps were combined and dried under a nitrogen stream. Samples were dissolved in 100 μl isooctane and kept at -20°C until analysis.

The following PLFAs were summed up to estimate bacterial biomass: i15:0, a15:0, 15:0, i16:0, 16:1ω7, 17:0, 18:1ω7t (Federle et al., 1983; Frostegård et al., 1993). The PLFA 18:2ω6,9 was used as marker for fungal biomass (Frostegård and Bååth, 1996). The ratio of 18:2ω6,9 to bacterial PLFAs was taken to represent the ratio of fungal to bacterial biomass in soil.

Whole cellular FAs of Collembola and spiders were saponified and methylated following the procedures given for the Sherlock Microbial Identification System (MIDI Inc., Newark, USA). Saponification of lipids was conducted in a sodium hydroxide-methanol solution (45 g sodium hydroxide, 150 ml methanol, 150 ml distilled water) at 100°C for 30 min, followed by acid methanolysis in HCl-methanol (325 ml 6.0 N hydrochloric acid, 275 ml methyl alcohol) at 80°C for 10 min. The FAMEs were extracted into hexane-methyl tertiary butyl ether (1:1) and washed with aqueous NaOH (10.8 g sodium hydroxide, 900 ml distilled water). The lipid-containing phase was then transferred to test tubes and stored at -20°C until analysis.

6.2.4 Analysis of the $^{13}C/^{12}C$ ratios of fatty acids

Gas-chromatography-combustion-isotope-ratio-monitoring-mass spectrometer system (GC-C-IRM-MS) was used to determine the isotopic composition of individual FAs in soil and animal samples. The system consisted of a gas chromatograph (6890 Series, Agilent Technology, USA) coupled via a Conflow II interface (ThermoFinnigan, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Germany). The performance and experimental details of this system are described in Richnow et al. (2003). A polar capillary column (50 m, 0.25 mm i.d., film thickness 0.25 mm) was used for the separation of FAMEs. The

polar column was chosen due to its better separation of unsaturated FAs compared to an unpolar column. For soil samples the GC split/splitless injector temperature was held at 250 °C. The split flow was 1:3 and helium was used as carrier gas. The temperature program was set as follows: 60 °C, 2 min isotherm, 20 °C min^{-1} to 140 °C; 2 °C min^{-1} to 160 °C, 5 min isotherm, 2 °C min^{-1} to 200 °C, 10 °C min^{-1} to 230 °C and held for 10 min. For animal samples GC split/splitless injector temperature was held at 250 °C. The split flow was 1:50 and helium was used as carrier gas. The temperature program was set as follows: 60 °C, 2 min isotherm, 10 °C min^{-1} to 140 °C, 5 °C min^{-1} to 200 °C, 10 °C min^{-1} to 250 °C, and held for 10 min. All samples were measured in at least three analytical replicates.

For the identification of FAs a fatty acid methyl standard (Suppelco 37 Component FAME Mix, Lot 47885-U) was employed. The standard was used to compare retention times of FAs for identification and to check the performance of the GC-C-IRM-MS system. To verify the correct identification of FAs (chain length and saturation) a range of samples was additionally analysed by GC-mass spectrometry. The carbon isotope composition is reported in δ-notation (‰) relative to Peedee belemnite marine limestone (PDB) according to:

$$\delta^{13}\mathrm{C}\,[‰] = (({}^{13}\mathrm{C}/{}^{12}\mathrm{C})_{sample}/({}^{13}\mathrm{C}/{}^{12}\mathrm{C})_{standard} - 1) \times 1000$$

Differences of ^{13}C between food source (A) and consumer (B) is described in terms of the difference in delta (d) values using the Δ notation, where $\Delta = \delta$B - δA. Positive Δ values indicate a relatively greater concentration of the isotope with the higher mass for B.

6.2.5 Analysis of ^{13}C/^{12}C ratios of plant material

For analysis of bulk ^{13}C/^{12}C signatures in maize and soybean tissues, samples of leaves and roots (n=3) were dried at 60°C, milled, weighed into tin capsules and stored in a desiccator until measurement. Isotope ratios were determined by a coupled system of an elemental analyser (NA 1500, Carlo Erba, Milan) and a mass spectrometer (MAT 251, Finnigan, Bremen). Stable isotope abundance is expressed using the δ notation with

$$\delta X\,[‰] = (R_{sample} - R_{standard})/R_{standard} \times 1000$$

with "X" representing ^{13}C, R_{sample} and $R_{standard}$ representing the ^{13}C/^{12}C ratios of the sample and standard, respectively. For ^{13}C Peedee belemnite marine limestone (PDB) was used as the standard.

6.2.6 Statistical analysis

Multivariate fitting of FA pattern and δ^{13}C in FAs was performed by MANOVA and significances are given according to Wilk's test. Differences of δ^{13}C in individual FAs between consumer and potential food source were subjected to Student's t-test. PLFA

data of soil samples were analysed using two factorial ANOVA. Statistics were carried out with STATISTICA 7.1 (StatSoft Inc., USA). Difference of $\delta^{13}C$ signatures in individual FAs were tested by GLM using SAS (SAS Institute Inc., Cary, USA).

6.3 Results

organism	treatment	F	treatment df	error df	P
Microorganisms (PLFA soil)					
	P	1.92	9	3	0.3213
	FS	26.02	9	3	**0.0107**
	PxFS	12.94	9	3	**0.0293**
P. fimata					
	P	4.04	3	1	0.3469
	FS	26.42	3	1	0.1418
	PxFS	1.27	3	1	0.5598
O. villosa					
	P	91.03	4	3	**0.0018**
	FS	10.89	4	3	**0.0393**
	PxFS	2.97	4	3	0.1991
P. agrestis					
	P	4.66	5	2	0.1861
	FS	1.74	5	2	0.4034
	PxFS	0.52	5	2	0.7610
M. acalypha					
	P	0.70	4	5	0.6252
	FS	4.47	4	5	0.0659
	PxFS	9.07	4	5	**0.0163**

Table 6.1:
MANOVA on the effect of crop plant (P; soybean, maize) and farming system (FS; conventional, bio-dynamic) on the composition of dominant FAs (proportion > 5%) of microorganisms, Collembola (*Protaphorura fimata*, *Orchesella villosa*) and spiders (*Pardosa agrestis*, *Mangora acalypha*) (FAs: n = 9, 4, 5, 4 for microorganisms, Collembola, *P. agrestis* and *M. acalypha*, respectively). Significant effects are in bold. F values according to Wilk's λ.

6.3.1 Fatty acid pattern

In soil 16 predominant PLFAs with a chain length between 14 and 18 carbon atoms were identified (data not presented). The pattern was significantly influenced by farming system and by the interaction of farming system and crop plant (Table 6.1). The total amount of PLFAs at the sites ranged between 2319 and 3782 nmol g^{-1} dry weight and was

higher in BIODYN compared to CONMIN treatments ($F_{1,11}$=4.69, P=0.053; Table 6.2). Based on marker PLFAs (see Materials and Methods) the amount of bacterial PLFAs was 1069 to 1694 nmol g^{-1} dry weight and of fungal PLFAs 265 to 519 nmol g^{-1} dry weight at the different sites. The latter increased distinctly in BIODYN treatments ($F_{1,11}$=16.46, P=0.002). Correspondingly, the ratio of fungal-to-bacterial PLFA was higher ($F_{1,11}$=6.06, P=0.0316) with 0.34 and 0.31 at BIODYN compared to 0.24 and 0.25 in CONMIN treatments, for maize and soybean, respectively.

The FA profile of Collembola comprised 15 predominant FAs with a chain length of 10 to 20 carbon atoms (data not presented). Most frequent were the FAs 16:0, 18:1ω9 and 18:2ω6,9. The FA composition of *O. villosa* was influenced by both crop plant and farming system (Table 6.1). The saturated FAs 16:0 and 18:0 had lower and the unsaturated FA 18:2ω6,9 higher proportions in soybean fields. The FA pattern of *P. fimata* was not affected by the different treatments.

Lipids of spiders contained 16 different FAs of a chain length of 10 to 20 carbon atoms, with 16:0, 18:1ω9 and 18:2ω6,9 as most frequent FAs (data not presented). The FA profiles of the web building *M. acalypha* significantly differed between farming systems, but the effect varied between crop plants (Table 6.1), whereas FA profiles of the cursorial *P. agrestis* were not significantly affected by experimental treatments.

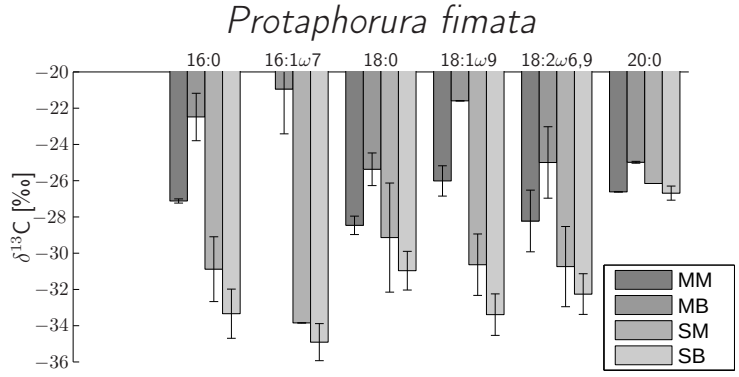
PLFA [nmol g^{-1} dry weight]	MM	MB	SM	SB
Total	2437±286	2885±1214	2319±728	3782±851
Fungal	268±60	388±132	265±56	519±77
Bacterial	1116±123	1255±641	1069±346	1694±465
Fungi/bacteria ratio	0.24±0.03	0.34±0.10	0.25±0.03	0.31±0.04

Table 6.2:
Amount (nmol g^{-1} dry weight ± s.d.) of total, fungal and bacterial phospholipid fatty acids (PLFAs) in soil samples from agricultural systems studied: **m**aize in conventional **m**ineral (MM) or **b**iodynamic farming system (MB); **s**oybean in conventional **m**ineral (SM) or **b**iodynamic farming system (SB).

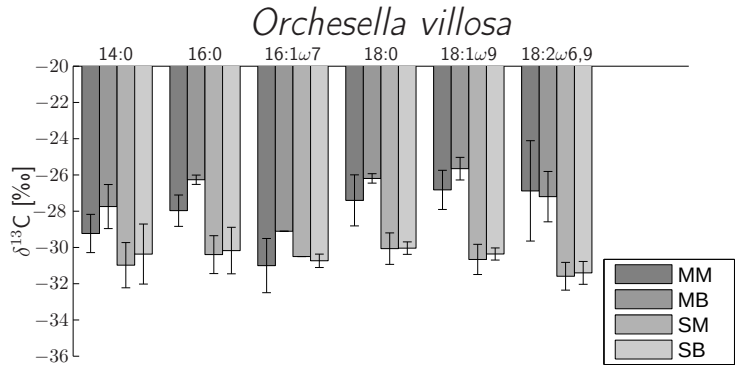
6.3.2 $^{13}C/^{12}C$ ratios in soil PLFAs and plant tissue

The $\delta^{13}C$ signatures of PLFAs extracted from the soil ranged from -24.1 to -32.4‰ in BIODYN and -25.1 to -35.0‰ in CONMIN under maize, and from -21.9 to -34.7‰ in BIODYN and -23.3 to -34.8‰ in CONMIN under soybean (data not presented). Differences in $\delta^{13}C$ in individual FAs between C_3 and C_4 crop fields were about 2‰. Signatures of $\delta^{13}C$ in PLFAs were significantly affected by crop plant and farming system (MANOVA; Table 6.3).

Signatures of $\delta^{13}C$ of leaves and roots of maize were -12.9‰±0.02 and -13.7‰±0.1, respectively. In soybean $\delta^{13}C$ signatures of leaves and roots were -27.2‰±0.1 and -26.6‰±0.2, respectively.



(a)



(b)

Figure 6.1:
Signatures of δ^{13}C of dominant fatty acids (‰± s.d.) in the Collembola *Protaphorura fimata* (a) and *Orchesella villosa* (b) as affected by crop plant and farming system: **m**aize in conventional **m**ineral (MM) or **b**iodynamic farming system (MB); **s**oybean in conventional **m**ineral (SM) or **b**iodynamic farming system (SB).

6.3.3 $^{13}C/^{12}C$ ratios in fatty acids of animals

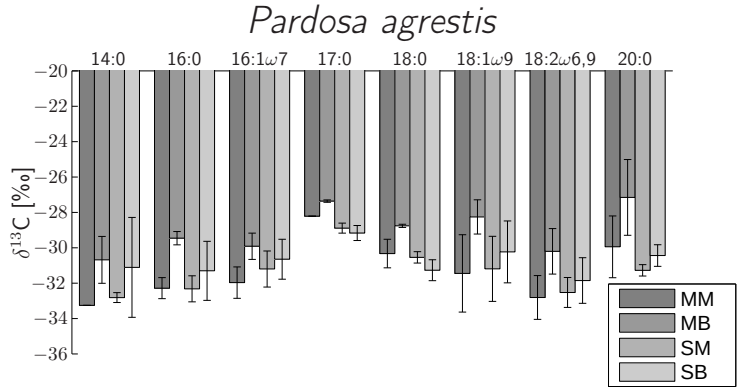
Collembola distinctly reflected the isotopic signal of the C_3 and C_4 crops. As indicated by MANOVA δ^{13}C signatures of frequent FAs from both *P. fimata* and *O. villosa* differed significantly between maize and soybean treatments (Table 6.3). The δ^{13}C of individual FAs in *P. fimata* in maize fields ranged from -26.0 to -28.5‰ in CONMIN and -21.0 to -25.4‰ in BIODYN treatments (Fig. 6.1(a)). The isotopic composition of FAs under soybean ranged from -26.2 to -33.9‰ and from -26.7 to -34.9‰ in CONMIN and BIODYN treatments, respectively. Generally, δ^{13}C signatures of FAs were highest in BIODYN maize, intermediate in the CONMIN maize and low in soybean treatments, with the ones in the CONMIN being slightly higher than in the BIODYN treatment. Differences in the δ^{13}C signature in *O. villosa* due to management practice were less prominent. In maize fields δ^{13}C of FAs ranged from -26.8 to -31.0‰ and from -25.7 to -29.1‰ in CONMIN and BIODYN treatments, respectively (Fig. 6.1(b)). In *O. villosa* under soybean crop δ^{13}C signatures of FAs ranged from -30.1 to -31.6‰ in CONMIN and -30.0 to -31.4‰ in BIODYN treatments.

In the cursorial *P. agrestis* the δ^{13}C signature of FAs did differ significantly due to an interaction of crop plant and farming system (Fig. 6.2(a), Table 6.3). Signatures of δ^{13}C under maize in the BIODYN treatment ranged from -27.2 to -30.7‰ and in the CONMIN treatment from -28.2 to -33.3‰ (Fig. 6.2(a)). Signature of δ^{13}C in FAs under soybean in CONMIN and BIODYN treatments ranged from -28.9 to -32.8‰ and from -29.2 to -31.9‰, respectively. However, in BIODYN maize fields the δ^{13}C signatures of 17:0 ($F_{3,5}$=14.0, P=0.0073) and 18:0 ($F_{3,6}$=9.85, P=0.0098) were significantly higher than in all other fields. The same was found as a trend in 15:0 ($F_{3,5}$=3.18, P=0.1228), 16:0 ($F_{3,6}$=3.27, P=0.1009) and 20:0 ($F_{3,5}$=4.32, P=0.0744). The isotopic composition of FAs in the web building *M. acalypha* did not significantly differ between systems; δ^{13}C signature ranged from -26.02 to -29.92‰, -26.21 to -31.61‰, -27.51 to -30.28‰ and -27.58 to -29.78‰ in CONMIN maize, BIODYN maize, CONMIN soybean and BIODYN soybean treatments, respectively (Fig. 6.2(b)).

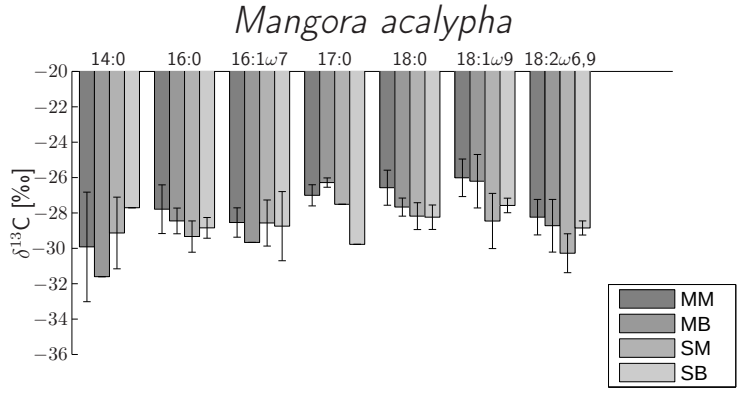
organism	treatment	F	treatment df	error df	P
Microorganisms (PLFA soil)					
	P	17.60	9	3	**0.0189**
	FS	7.16	9	3	0.0662
	PxFS	1.88	9	3	0.3284
P. fimata					
	P	116.40	4	2	**0.0085**
	FS	1.28	4	2	0.4809
	PxFS	16.04	4	2	0.0595
O. villosa					
	P	38.80	4	6	**0.0001**
	FS	2.00	4	6	0.1877
	PxFS	1.16	4	6	0.3956
P. agrestis					
	P	9.23	5	2	0.1009
	FS	2.03	5	2	0.3703
	PxFS	37.05	5	2	**0.0265**
M. acalypha					
	P	3.26	4	5	0.0957
	FS	1.44	4	5	0.3277
	PxFS	1.48	4	5	0.3180

Table 6.3:

MANOVA on the effect of crop plant (P; soybean, maize) and farming system (FS; conventional, bio-dynamic) on the δ^{13}C in dominant FAs (proportion > 5%) of microorganisms, Collembola (*Protaphorura fimata*, *Orchesella villosa*) and spiders (*Pardosa agrestis*, *Mangora acalypha*) (FAs: n = 9, 4, 5, 4, for microorganisms, Collembola, *P. agrestis* and *M. acalypha*, respectively). Significant effects are in bold. F values according to Wilk's λ.



(a)



(b)

Figure 6.2:
Signatures of δ^{13}C of dominant fatty acids (‰± s.d.) in the spiders *Pardosa agrestis* (a) and *Mangora acalypha* (b) as affected by crop plant and farming system: **m**aize in conventional **m**ineral (MM) or **b**iodynamic farming system (MB); **s**oybean in conventional **m**ineral (SM) or **b**iodynamic farming system (SB).

6.3.4 Δ^{13}C of FAs between animals and potential food sources

Collembola and their food resources

Recent studies (Haubert et al., 2006, see Chapter 4; Ruess et al., 2005a) ascribed individual FAs to food resources in Collembola. The proposed dietary trophic markers are 16:1ω7 for bacteria, 18:1ω9 for leaves and 18:2ω6,9 for fungi. We compared the isotopic composition of these FAs between Collembola consumers and their potential food sources. C_3 and C_4 crop plants strongly impacted the isotopic shift in the euedaphic *P. fimata* (Fig. 6.3(a)). Under soybean 16:1ω7 and 18:1ω9 in *P. fimata* were depleted compared to PLFAs of potential diet with a Δ^{13}C ranging from -2.1 to -7.0‰, and a significant effect of farming system for 18:1ω9 (P=0.035, t-test). In contrast, δ^{13}C of 18:2ω6,9 was similar in *P. fimata* and the corresponding fungal PLFA in both CONMIN and BIODYN systems. In maize fields FAs of *P. fimata* were generally enriched in ^{13}C compared to bacterial, plant or fungal marker PLFAs by 1.8 to 10.0‰, except for 18:2ω6,9 in CONMIN fields in which δ^{13}C signatures were similar in Collembola and fungi.

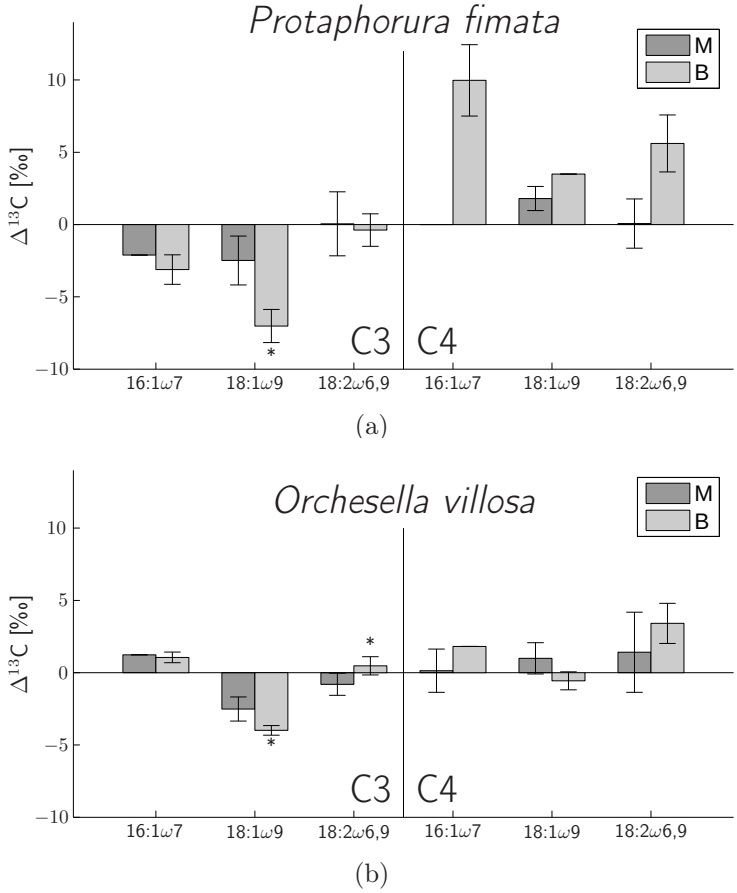


Figure 6.3:
Differences between δ^{13}C signatures in Collembola (*Protaphorura fimata* (a) and *Orchesella villosa* (b)) and marker FAs (Δ^{13}C ± s.d.) for bacterial (16:1ω7), leave tissue (18:1ω9) and fungi (18:2ω6,9) as potential food resources under different crop plants and farming systems: **m**aize in conventional **m**ineral (MM) or **b**iodynamic farming system (MB); **s**oybean in conventional **m**ineral (SM) or **b**iodynamic farming system (SB). $*$ indicate significant difference (t-test, $P<0.05$).

In the euedaphic *O. villosa* from soybean fields δ^{13}C signatures of the FAs 16:1ω7 and 18:2ω6,9 were similar to those of PLFAs extracted from the soil (1.2 and 1.1‰, and -0.8 and 0.5‰ in CONMIN and BIODYN fields, respectively; Fig. 6.3(b)). In *O. villosa* 18:1ω9 was depleted in δ^{13}C compared to the potential plant resource by 2.5 to 4.0‰, and the shift significantly differed between farming systems (P=0.017, t-test). Under maize the δ^{13}C signature of the FA 16:1ω7 was similar between *O. villosa* and soil PLFA in the CONMIN treatment (Δ^{13}C= 0.14‰) and those of 18:1ω9 in both the CONMIN (Δ^{13}C = -0.57‰) and BIODYN fields (Δ^{13}C = 0.99‰). In contrast, the δ^{13}C signature of the FA 18:2ω6,9 in *O. villosa* and in fungi in soil were more different with Δ^{13}C varying from 1.4 to 3.4‰.

Spiders and Collembola prey

Signatures of δ^{13}C of corresponding FAs in the cursorial spider *P. agrestis* and Collembola differed between fields planted with C_3 and C_4 crops (Fig. 6.4). FAs from *P. agrestis* in

maize fields had much lower δ^{13}C signatures than FAs from both Collembola species with the difference being as high as -9.0‰. In soybean fields differences in δ^{13}C signatures between *P. agrestis* and Collembola were more variable ranging between -1.9 and +4.3‰. In *P. agrestis* and *O. villosa* from soybean fields δ^{13}C signatures of FAs differed little, in particular in 16:1ω7 (-0.7 to 0.1‰) and 18:1ω9 (-0.5 to +0.1‰) (Fig 6.4(b)). In contrast, differences in δ^{13}C signatures of FAs of *P. agrestis* and *P. fimata* were more pronounced except for 18:0 in the BIODYN treatments (Δ^{13}C = -0.3‰ ±0.6) (Fig 6.4(a)). Farming system significantly affected the shift in δ^{13}C for 16:0 and 18:0 (P=0.030 and P=0.048, respectively, t-test).

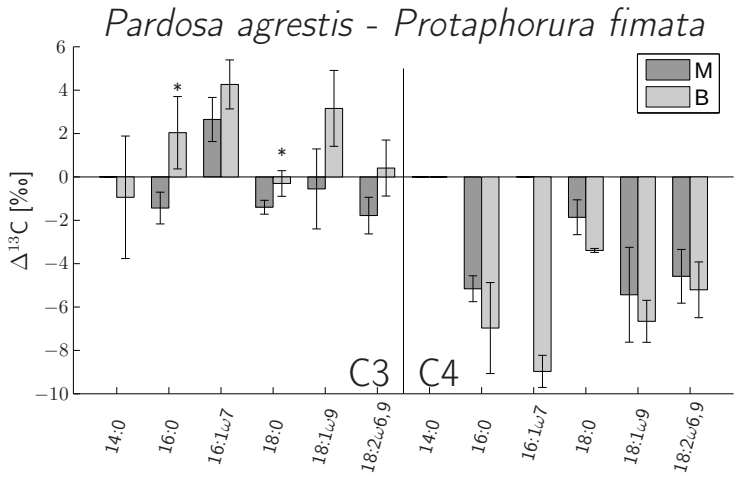
Differences in δ^{13}C signatures of corresponding FAs in the web-building spider *M. acalypha* and Collembola ranged from -5.9 to +4.3‰ (data not presented). Only the signatures of 16:0 in *M. acalypha* and *O. villosa* differed little in the CONMIN treatment of maize (Δ^{13}C = 0.2‰ ±1.4).

6.4 Discussion

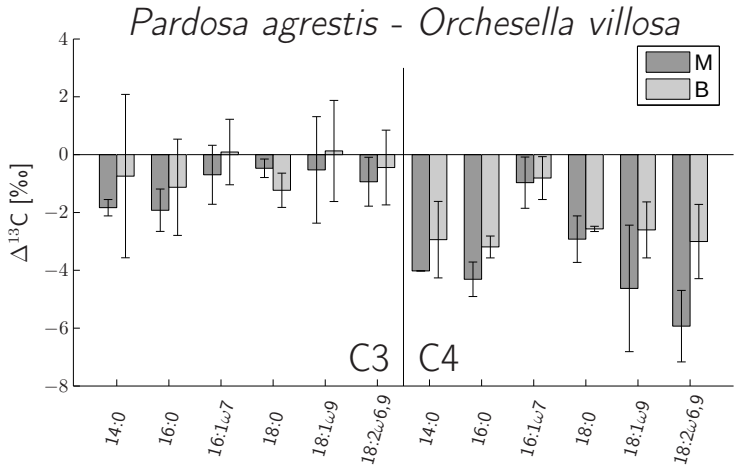
6.4.1 Microorganisms

Phospholipids are a component of all living cells and as they decompose rapidly after cell death, they are good indicators of the composition of the living soil microbial biomass (Bailey et al., 2002). PLFAs characteristic for specific microorganisms can be used to assess their occurrence in soil (Frostegård and Bååth, 1996; Zelles, 1999; Bååth and Anderson, 2003). We investigated the structure of the microbial community in organic (BIODYN) and conventional (CONMIN) farming systems under two different crop plants. BIODYN led to an increase in soil microbial PLFA, most prominent those of fungi. Previously, it has been reported that microbial biomass C and N in organic systems exceeds that in conventional systems (Fließbach and Mäder, 2000). The review of Mäder et al. (1996) suggests that microbial activity (dehydrogenase, catalase) in organic (+30-70%) and biodynamic (+40-90%) agricultural systems generally exceeds that of systems receiving mineral fertiliser only. The ratio of fungal-to-bacterial PLFA was higher in BIODYN than in CONMIN systems, but was not affected by the crop plant. The organic fertilizers applied in the BIODYN system likely were responsible for the shift in the fungi-to-bacteria ratio towards fungi, since fungi have been found to benefit from farmyard manure as compared to mineral fertilizer (Smith et al., 2003). Correspondingly, PLFA patterns indicate that the community composition of soil microorganisms is affected by agricultural practice (Buyer and Drinkwater, 1997; Gunapala and Scow, 1998; Carpenter-Boggs et al., 2000).

Soil carbon in croplands derives mostly from roots and not from aboveground plant litter (Balesdent and Balabane, 1996). Vegetation shifts from C_3 to C_4 plants cause root induced changes in soil organic C, water soluble organic C and microbial C (Liang et al., 2002). At the investigated agricultural sites the isotopic signature of the C_4 crop was reflected in soil microorganisms in the first year of cultivation of maize by an increase in δ^{13}C in PLFAs of about 2‰. Considering the difference in δ^{13}C of 14 ‰ between C_3 and



(a)



(b)

Figure 6.4:
Differences between δ^{13}C (Δ^{13}C $\pm$ s.d.) in FAs between the cursorial, epigeic spider *Pardosa agrestis* and the Collembola *Protaphorura fimata* (endogeic) (a) or *Orchesella villosa* (epigeic) (b) under different crop plants and farming systems:**m**aize in conventional **m**ineral (MM) or **b**iodynamic farming system (MB); **s**oybean in conventional **m**ineral (SM) or **b**iodynamic farming system (SB). $*$ indicate significant difference (t-test, $P<0.05$).

C_4 plants this response was small. Bruulsema and Duxbury (1996) reported that 23% of the microbial biomass was derived from maize over the course of a single growing season. Liang et al. (2002) observed maize carbon to build 48% of the microbial biomass and 12% of the soil organic carbon 110 days after sowing in a greenhouse study. Long term surveys (39 years) estimated 23-46% of the carbon in microbial biomass to originate from maize after conversion from rye (John et al., 2003, 2004). In contrast, the low C_4 signal in the present study indicates a smaller input of maize derived C and a considerable use of the soil C_3 pool by the microbial community.

6.4.2 Collembola

Collembola feed on a wide spectrum of food sources, including bacteria, fungi, algae, plant litter, living plant tissue and animals (Rusek, 1998). Fungi are regarded as the most important food source (Chen et al., 1995; Klironomos and Kendrick, 1995). Recently, the lipid composition of Collembola has been used to trace feeding strategies (Haubert et al., 2006, see Chapter 4; Ruess et al., 2004, 2005a; Chamberlain et al. 2005). At the investigated agricultural sites the FA pattern of the euedaphic *P. fimata* was not affected by crop plant and farming system, suggesting that the major food resources have similar FA profiles across sites. Generally, fungi synthesise their FAs de novo from carbohydrates, i.e. trophic transport of FAs from plant litter or root exudates to fungi is negligible (Pfeffer et al., 1999). Therefore, the FA profiles of fungal grazers, such as *P. fimata*, are less dependant on the aboveground plant community, which is in line with our results.

In contrast to microorganisms, the C_4 signature in individual FAs of the decomposer fauna, represented by Collembola, was distinct with 4 to 10 delta units difference between maize and soybean fields. This indicates that major carbon sources of the animals derived from the current plant crop. They either selectively assimilated fresh C_4-C from residues or fed on microorganisms, degrading maize residues. The FAs of the euedaphic *P. fimata* were enriched in δ^{13}C by up to 10 delta units in C_4 systems. The C_3/C_4 signal was distinctly reflected in the metabolically linked FAs 16:0, 18:0, 18:1ω9 and 18:2ω6,9. This indicates biosynthesis from a δ^{13}C enriched FA 16:0, the major pool in the FA pathway, receiving carbon from various dietary components, such as FAs, carbohydrates and proteins. *Onychiurus* species (like *P. fimata*) are regarded as predominant fungal feeding (Visser et al., 1987; Bardgett et al., 1993; Sadaka-Laulan et al., 1998; Maraun et al., 2003; Chahartaghi et al., 2005), however, Filser (2000) suggested that *Protaphorura armata* also feeds on roots. Increased δ^{13}C signatures of *P. fimata* in our study therefore likely resulted from consumption of fungi decomposing maize residues or additionally from assimilation of carbon derived directly from plant roots.

In animals the intramolecular isotopic distribution in FAs is assumed to be a function of the relative de novo synthesis and the assimilation of precursors from the diet (DeNiro and Epstein, 1977). FAs with dietary routing are expected to display a δ^{13}C comparable to FAs in the food source, whereas when synthesised from dietary carbohydrates (i.e. via

acetyl coenzyme A) they are depleted in δ^{13}C (Gannes et al., 1998; McCutchan et al., 2003). Differences in δ^{13}C of FAs in consumer and resources therefore reveal information on trophic links.

In *P. fimata* the Δ^{13}C in PLFAs from soil and related Collembola FAs varied strongly between C_3 and C_4 systems. However, no shift in Δ^{13}C was observed for the fungal marker linoleic acid, except in BIODYN maize fields. This indicates dietary routing of linoleic acid into tissues of *P. fimata* and predominant fungal feeding, which is in line with the other results of this study.

FA pattern of the epigeic *O. villosa* was predominantly affected by crop plant, and less by farming system. Most prominent was the increase in linoleic acid (18:2ω6,9) in *O. villosa* in soybean fields. This indicates consumption of plant tissue, as soybean lipids comprise more than 50% of linoleic acid (Sato et al., 2004). With a maximum shift in δ^{13}C of FAs of up to 4 delta units between maize and soybean the epedaphic species *O. villosa* reflected the isotopic composition of the current crop plant less than the euedaphic *P. fimata*. The combined utilisation of old (C_3) and new (C_4) carbon sources in maize fields influences the signal in the Collembola, that was also found by Albers et al. (2006), where maize-born carbon never exceeded 50% in meso- and macrofauna animals during 2 years of maize cropping.

For *O. villosa* the shift in δ^{13}C in 16:1ω7 between consumer and potential diet was small, which may point to the ingestion of bacteria, however, 16:1ω7 may also be derived from plant tissue (Ruess et al., 2005b). In soybean fields no shift in signature of 18:2ω6,9 occurred, suggesting fungal feeding, but in fact, soybean leaves are also rich in linoleic acid (Sato et al., 2004). We did not measure δ^{13}C of FAs in plant litter, but according to Hobbie and Werner (2004) lipids are depleted by 4-6‰ in C_3 plants relative to bulk tissue. Complementary, FAs in soybean litter range between 31 and 33‰, which corresponds to the Δ^{13}C of linoleic acid in *O. villosa*. Consumption of plant tissue by this epedaphic species therefore is likely. Additionally, under maize crop the Δ^{13}C in the marker FA for plants (18:1ω9) indicated dietary routing into consumer lipids. In sum, the results suggest plant litter as predominant food source in *O. villosa* and this has been suggested for *Orchesella* species before (Varga et al., 2002; Berg et al., 2004; Chahartaghi et al., 2005; Albers et al., 2006).

6.4.3 Spiders

The prey spectrum of spiders in European crop fields mainly comprises of Collembola, Diptera and Homoptera (Nyffeler et al., 1994; Nyffeler and Sunderland, 2003). The FA composition of the dominant spiders at the investigated fields, *P. agrestis* and *M. acalypha*, was generally not affected by crop plant or farming system. This suggests that the FA profile of the major prey of both spiders differs little across sites. However, for *M. acalypha* MANOVA proved a significant interaction of crop plant and farming system, indicating a spacially more restricted prey spectrum in this web building species compared to the cursorial *P. agrestis*.

The δ^{13}C signature of FAs of spiders differed little between maize (-26.02 to -33.26‰) and soybean fields (-27.51 to -32.82‰). However, the δ^{13}C signal of *P. agrestis* was slightly shifted towards the C_4 crop in the BIODYN maize field, suggesting that it consumed prey from the maize field.

Comparing corresponding FAs of *P. agrestis* and Collembola between C_3 and C_4 plant systems, the depletion in δ^{13}C between species in maize fields by -0.97 to -8.97 delta units does not suggest a predator-prey relationship. In soybean fields difference ranged between -1.92 and +4.26‰, with a very heterogeneous pattern in comparison to *P. fimata*, indicating that *P. agrestis* did not feed on this euedaphic Collembola species. In contrast, the difference in δ^{13}C between related FAs in *P. agrestis* and *O. villosa* was less than 2‰ for 16:1ω7 and 18:1ω9. This suggests epigeic Collembola as potential prey for this cursorial spider. Consistent with this finding the diet of *Pardosa* species has been found to comprise up to 43% of Collembola (Nentwig, 1986; Nyffeler, 1999; Bilde, et al. 2000, Oelbermann et al. 2006.). The shift in δ^{13}C of FAs of *P. agrestis* and *O. villosa* was close to zero particularly at BIODYN fields. Potentially, *P. agrestis* were more stationary in these fields, because bio-organic fertilisation results in a higher abundance of epigeic Collembola (Primentel and Warneke, 1989) and wolf spiders are known to change their area of prey capture actively in relation to prey density (Samu and Bíró, 1993). Additionally, due to lack of herbicide applications more weeds were present in BIODYN fields and therefore more microsites and shelter for spiders. Wolf spiders respond to differences in soil texture and management practice (Marshall and Rypstra, 1999), with only short stops in open surfaces but more time spent in structurally diverse habitats (Samu et al., 2003). Presumably, despite the small size of the plots lycosids spiders formed resident populations in the experimental fields.

In contrast to *P. agrestis*, the δ^{13}C pattern in FAs of *M. acalypha* was little related to δ^{13}C in corresponding FAs of Collembola. The shift in δ^{13}C ranged from -5.93 to 4.26‰, and was close to zero only for the FA 16:0 in the CONMIN maize field. These differences suggests no close predator-prey relationship, but similarity in FA 16:0 suggests that body carbon of both *O. villosa* and *M. acalypha* originate from the same basal resources. The web building *M. acalypha* colonises vegetation strata and primarily consumes herbivores, such as Homoptera, Diptera and Coleoptera (Nyffeler, 1999), rather than decomposers, such as Collembola, and this is supported by our findings.

6.5 Conclusions

Isotopic relationships between the dietary input and the consumer tissue is complicated by a variety of factors such as biosynthetic pathways, metabolic status or diet quality (Haubert et al., 2005, see Chapter 3; Gearing 1991; Focken and Becker 1998; Gaye-Siessegger et al 2004a,b; Scheu and Folger, 2004). Results of this study demonstrate that δ^{13}C signatures of individual FAs in potential prey (diet) and consumers allow to identify carbon fluxes and trophic links. Euedaphic Collembola were found to depend strongly on local soil carbon resources from the current crop plant, whereas the diet of

epedaphic species is based on carbon from current crop plants but also on soil carbon originating from former crop plants. The trophic shift of FAs identified *P. fimata* as fungal feeder, and *O. villosa* as predominantly living on plant material. The cursorial epedaphic spider *P. agrestis*, but not the orb web species *M. acalypha*, was linked via its prey to the decomposer food web. Corresponding δ^{13}C in FAs indicated the Collembola species *O. villosa* as major prey of *P. agrestis*. In conclusion, the combination of stable carbon isotope analysis with assigned trophic marker FAs gave detailed insight into food-web interactions under natural field conditions. The method therefore is a promising tool for disentangling trophic interrelationships in complex food webs, such as those in soil.

Chapter 7

General Discussion

Soils harbour a great diversity of organisms which are linked in a complex web of trophic interactions. Over the last decade specific biochemical markers were used as tool to identify trophic relationships in aquatic and terrestrial habitats. However, diet, metabolic status and environmental conditions may affect the resolution of these biomarkers. Defined laboratory experiments are needed to verify the power of routinely employed approaches such as stable isotope analysis, but also new advances such as fatty acid profiling. Moreover, their application under field conditions has to be tested.

7.1 Stable isotope ratios as signal for trophic interactions

Gannes et al. (1997) proposed stable isotope analysis to be a powerful tool in food web ecology. The present study contributes to their call for more laboratory experiments to assess factors which hamper the interpretation of isotopic ratios. Remaining uncertainty in the isotopic fractionation can affect estimates of the proportionate contribution of food sources or assigned trophic positions (McCutchan et al., 2003; Gaye-Siesegger et al., 2003, 2004a,b). Food web studies commonly apply a mean shift of +1‰ for Δ^{13}C and of +3.4‰ for Δ^{15}N with each trophic step. The performed experiments showed that factors like starvation, food quality and developmental stage of Collembola led to variations of -1.0 to -3.3‰ for δ^{13}C and 2.1 to 6.3‰ for δ^{15}N between trophic levels. This clearly indicates that differences in animal physiology can result in a fractionation comparable to trophic level shifts. However, the implication of our laboratory findings to the field remains to be explored. We have investigated few feeding links, and the postulated mean trophic level fractionation for carbon and nitrogen (1‰ and 3.4‰, respectively) may still be a valid approximation when applied on entire food webs with multitrophic pathways and many species (Post, 2002). In field experiments, this method gave good estimates on trophic relations in Collembola and mite populations (Schneider et al., 2004; Chahartaghi et al., 2005). Nevertheless, the drawbacks indicated by the present study should be taken into account when interpreting stable isotope data.

7.2 Fatty acids as trophic biomarkers

Fatty acid (FA) analysis has been applied in food web studies in aquatic ecosystems (Meziane et al., 1997; Ederington et al., 1995; Gladyshev et al., 1999, 2000; Navarrete, 2000) and was recently transferred to soil systems (e.g. Chen et al., 2001; Chamberlain et al., 2005; Ruess et al., 2002, 2004). For the use of FAs as trophic biomarkers, pattern of neutral lipid fatty acids (NLFAs) are important, as dietary FAs are predominantly incorporated into deposit fat of the consumer, and NLFAs therefore may reflect the food source. In contrast, phospholipid fatty acids (PLFAs) are the main constituent of cell membranes and are closely related to physiological needs. To verify the application of FA analysis for food web studies, factors which influence the lipid composition of organisms were investigated.

7.2.1 Food quality and depletion

Food depletion of Collembola led to a decrease in the total amount of neutral lipids, whereas the relative proportion of most NLFAs did not change. FAs were metabolised indiscriminately, which suggests that the occurrence of biomarker FAs will not be affected directly by starvation. Similar results were obtained for other insects, such as Triatominae (Heteroptera) (Canavoso et al., 1998). In line with other studies (e.g. Simpson and Abisgold, 1985; Karowe and Martin, 1989; Simpson and Simpson, 1990; Lavy and Verhoef, 1996) we found that diet with a low quality (i.e. high C/N ratio) resulted in compensatory feeding to satisfy the need for nitrogen and subsequently to an increased uptake of carbon and its deposition in neutral lipids. That led to an altered FA pattern depending on the C/N ratio of the fungal food source. However, the FA 18:2ω6,9 which is proposed as marker for fungal consumption (Ruess et al., 2005b) was still the most abundant in Collembola NLFA (47 - 76%) indicating ingestion of fungi.

In comparison to neutral lipids, the composition of PLFAs in Collembola was only slightly altered by food quality or starvation. This is likely due to the function of PLFAs as structural components of membranes, with no relation to energy metabolism. However, PLFA patterns may change in the long-term due to altered metabolism caused by food deprivation. Additionally, the amount of PLFAs increased with increasing nitrogen content of the fungal diet. Better food quality likely resulted in an increased growth rate and egg production (Booth and Anderson, 1979; Lavy and Verhoef, 1996), i.e. a need for cell development and consequently more PLFAs. In sum, the results suggest that the influence of dietary lipid composition on the pattern of consumers may be altered by their metabolism and physiological status, diminishing the signal of individual biomarker FAs.

7.2.2 Environmental temperature and development stage of Collembola

Animals can adapt to variations in temperature conditions mainly by altering the FA composition in membranes (Hazel, 1995). Most studies refer to alteration of PLFAs whereas

changes in NLFAs are not well documented. Generally, the amount of unsaturated PLFAs in animals increases with declining environmental temperature (Hazel, 1995; Abu Hatab and Gaugler, 1997). Besides, the involvement of eicosanoids in physiological processes may affect the lipid composition of Collembola. Certain FAs, such as the proposed biomarker 18:2ω6,9, are precursors for the hormonal active C20 polyunsaturated FAs (Stanley-Samuelson and Nelson, 1993), which are important for reproduction, mediate cellular immune response and are involved in temperature regulation (Stanley-Samuelson and Nelson, 1993; Toolson et al., 1994). The performed experiments showed that temperature or development stage of Collembola mainly influenced the amount of NLFAs and not their general occurrence. The FA 18:2ω6,9, which is the marker for fungal food, was present in high amounts (over 20% of all FAs) at all temperatures and life stages. The proportion was higher than in Collembola feeding on other diets (2 - 14%). Therefore, high amounts of 18:2ω6,9 in the FA profile of Collembola can serve as a valid indicator for fungi as main food source, independently of environmental temperature or development stage.

7.2.3 Fatty acids indicating bacterial diet

Collembola feeding on bacteria can affect the composition of the soil microbial community by their grazing activity or by a selective gut passage and dispersal of bacteria (Thimm et al., 1998; Hoffmann et al., 1999). Bacteria contain several FAs that are not routinely biosynthesised by Collembola and therefore can be used as biomarkers for ingestion of bacterial food sources. Methyl-branched (iso, anteiso) FAs are characteristic for gram-positive bacteria and cyclopropylrings are predominantly found in gram-negative bacteria (Welch, 1991; Zelles, 1999). Collembola on bacterial diet contained these marker FAs in their NLFA profiles. In contrast, the bacterial derived FAs were not found in the lipids of Collembola feeding on other diets such as fungi or plant leaves (Haubert et al., 2004, see Chapter 2; Ruess et al., 2004, 2005a,b). This indicates that marker FAs can be used to trace bacterial diet of Collembola. Estimates of the NLFA/PLFA ratio, body weight, δ^{13}C and δ^{15}N gave additional information on the metabolic status of Collembola and the food quality of bacteria. *Enterobacter aerogenes* was of low, *Bacillus megaterium* and *Pseudomonas putida* of high food quality for Collembola.

7.2.4 Fatty acids as indicators for feeding strategies

My experiments and those of colleagues revealed FA biomarkers for different Collembola diets. FAs with branched chain (i14:0, i15:0, a15:0, i17:0) or cyclopropylrings (cy17:0) indicate feeding on gram-negative and gram-positive bacteria, respectively (Haubert et al., 2006, see Chapter 4). With nematodes as food source the FA 20:1ω9 was detected in the lipids of Collembola (Ruess et al., 2004, 2005b). Both FA markers for bacteria and nematode food occurred only when Collembola were fed on the specific diet, but were lacking when maintained on other food resources. This suggests that Collembola do not synthesise these FAs, and deposition in the NLFAs is dependant on the diet. For

consumption of plant leaves and fungi biomarker FAs were assigned due to their frequency in Collembola lipids. The amount of 18:2ω6,9 was always high (above 20%) in individuals feeding on fungi. Animals fed leaves had a greater amount of 18:1ω9 (39.3%) compared to Collembola on other diets (26-29%; Ruess et al., 2005b).

On the other hand Chamberlain et al. (2005) found high amounts of 18:1ω7 with up to 18% in Collembola feeding on nematodes compared to only 8% in Collembola on a leave, algae or fungal diet. Besides the 18:1ω7, they ascribed the FA i15:0 and i17:0 as marker for nematode food. This is not in line with my results, where these FAs were ascribed as biomarkers for bacterial diet. However, Chamberlain et al. (2005) used bacterial-feeding nematodes for their experiments, i.e. the origin of these branched FAs is likely based on the bacterial diet of nematodes.

In sum, the above discussed studies assigned marker FAs by occurrence or by increased frequency in the NLFAs of Collembola for predominant feeding strategies. Ruess et al. (2005b) performed first successful investigations of Collembola populations in the field. They compared three deciduous forest stands and assigned broad ecological groups (bacterivores, fungivores, herbivores, predators) by applying the FA biomarkers ascribed in laboratory experiments. Collembola diet indicated by FA markers were generally in accordance with existing knowledge on food resources and feeding modes. Therefore, FA analysis proved to be a valuable tool to investigate soil food webs. Further experiments are needed to define dietary biomarkers in other soil animals and to apply this method to whole food webs in the field.

7.3 Carbon stable isotope ratios of fatty acids

Compound-specific isotope analysis of FAs was first applied in Collembola feeding experiments by Chamberlain et al. (2004) and Ruess et al. (2005a). The advantage of this method is that the δ^{13}C signature of specific dietary marker FAs is gained. Similar $^{13}C/^{12}C$ ratio in the same FA in consumer and potential diet indicates trophic transfer and routing into the FA of consumer's tissue. On the contrary, different signatures indicate other food sources as the proposed or de novo synthesis of FAs from other carbon sources. In addition to analysis of FA patterns only, the determination of the origin of marker FAs by carbon isotopic signals results in a greater resolution, an important fact when investigating complex food webs.

The work of Chamberlain et al. (2004) and Ruess et al. (2005a) was performed in laboratory microcosms. In my experiments this approach was applied the first time to the field, using the natural label of C_3 and C_4 plants to trace the carbon flow in an agricultural food web. Determination of δ^{13}C in individual FAs of animals and food sources revealed that euedaphic Collembola like *Protaphorura fimata* depend strongly on local soil carbon from the current crop plant, whereas the epedaphic *Orchesella villosa* consumes carbon from current but also from former crop plants. Differences in δ^{13}C in single FAs between consumer and potential diet assigned *P. fimata* as fungal feeder and *O. villosa* as predominantly living on plant material. The cursorial spider *Pardosa agrestis*

was linked to the soil food web presumably via *O. villosa* as major prey. In contrast, the orb web spider *Mangora acalypha* was not dependant on soil organisms as prey. The application of compound-specific FA analysis, especially of assigned marker FAs, was in line with known facts about animal feeding strategies in agricultural soils. It is therefore a promising tool to investigate unknown trophic links in soil food webs and presumably will give detailed insight into feeding strategies and predator-prey relationships.

7.4 Prospects

Soil systems differ considerably from aboveground systems, particularly because of the unusual high diversity of species despite an apparent lack of niches of the species. Trophic niche differentiation may lead to reduced competition between species and may therefore explain the high diversity of soil animals (Anderson, 1975). However until now most studies gave strong evidence that soil food webs consist of associations of organisms whose trophic relationships are unspecific and also variable in time (Scheu, 2002; Scheu and Setälä, 2002). Methods investigated in the present work can help to unravel the "enigma" of high diversity of decomposer animal species in soil (Maraun et al., 2003). For following on these experiments, the application of FA analysis should be extended. It can now be broadened to other soil animal groups, besides Collembola and also to different soil depths, seasons and habitats. Specific FA markers for dominant taxa and functional groups have to be assessed in laboratory feeding experiment and subsequently have to be verified under field conditions. Particularly tempting is the use of compound-specific FA analysis in systems with different labelled carbon sources to investigate trophic relationships. Overall, for the first time fatty acid biomarkers provide, as documented in this study, a valuable tool for analysing complex trophic interrelationships and now can be applied to entire food webs under natural field conditions.

Chapter 8

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Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Hilfsmitteln angefertigt habe. Ich habe noch keinen weiteren Promotionsversuch unternommen. Die in dieser Arbeit dargestellten Ergebnisse basieren auf den von mir in diesen Versuchen erhobenen Daten.

Darmstadt, den 21.April 2006

(Dominique Haubert)